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Polychaetes (*Perinereis helleri*) reared in sand beds filtering nutrients from shrimp (*Penaeus monodon*) culture ponds can transiently carry IHHNV

Siyi Liu[a], Min Rao[b], Jeff A. Cowley[b], Jess A.T. Morgan[c], Andrew C. Barnes[a], Paul J. Palmer[c,*]

[a] The University of Queensland, School of Biological Sciences, St Lucia, QLD 4072, Australia
[b] Aquaculture, CSIRO Agriculture & Food, Queensland Bioscience Precinct, 306 Carmody Road, St. Lucia, QLD 4067, Australia
[c] Queensland Department of Agriculture and Fisheries, Ecosciences Precinct, 41 Boggo Road, Dutton Park, QLD 4102, Australia

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ABSTRACT

A polychaete-assisted sand filter (PASF) system has been developed to help remove nutrients from aquaculture pond wastewater whilst also producing polychaetes that are highly prized as bait by recreational anglers and as a dietary supplement to improve the fecundity of shrimp broodstock. Whilst rearing polychaetes in PASF beds offers potential to reduce impacts of sourcing them from the wild, the use of wastewater from ponds rearing shrimp such as *Penaeus monodon* will present a biosecurity risk of viruses being transferred to, and potentially amplified in, the worms. To assess such risks for transmitting infectious hypodermal and haematopoietic necrosis virus (IHHNV), groups of 3 or 4 PASF beds seeded with sand worm (*Perinereis helleri*) juveniles were supplied with wastewater from ponds of *P. monodon* with either high-load or low-load IHHNV infections. TaqMan real-time qPCR identified low loads of IHHNV ($\leq$878 IHHNV DNA copies 200 ng^{-1} TNA) in most worms from PASF beds supplied wastewater from the high-load pond. IHHNV was either not detected or detected at the qPCR test sensitivity limits in worms from beds supplied wastewater from the low-load pond. Purging harvested worms of their gut contents in clean filtered seawater for 2 days significantly reduced IHHNV loads. Reverting PASF beds to clean seawater for 8 weeks before harvest also significantly reduced worm loads of IHHNV. Daily additions of a commercial probiotic to the sand bed surface for 4 weeks prior to clean seawater application provided no discernible benefit to IHHNV clearance. While clearly demonstrated to be capable of carrying IHHNV, the remediation measures examined suggest potential to ameliorate the infection transmission risks of *P. helleri* reared in PASF beds supplied with shrimp pond wastewater as a nutrient source.

1. Introduction

Perinereis helleri (*Polychaeta*, *Nereididae*) is an omnivorous marine sandworm distributed widely throughout the Indo-Pacific region. Specimens have been recorded from coastlines in northern Australia (New South Wales to Western Australia), the Philippines, Indonesia, Ambon Reef, India, Chile, the Marshall Islands, Hawaii and the Ryukyu (Okinawa) Islands of Japan (Hutchings et al., 1991; Reish, 1956; Kohn and Orians, 1962; Okuda, 1940). They are burgundy-brown in colour and can grow to 200 mm (or more) in length and 2 g in weight depending on habitat and feed sources. Between their head and tail they have up to 139 morphologically identical segments (Palmer, 2010), each with parapodia for locomotion and respiration and a set of essential body organs. If damaged or truncated, this repetition of essential body components often facilitates their survival and ability to grow a new tail. The worm head possesses an eversible proboscis with a raptorial pair of jaws used to grab food particles and made of zinc and a toughened histidine-rich protein (Broomell et al., 2008), and a muscular pharynx lined with chitinous paragnaths that assists in burrowing and feeding. The head feeding appendages supply matter to an oesophagus that transitions to an intestine extending to an anus at the tail tip.

Perinereis helleri inhabits a wide variety of shelly rubble and sandy foreshores in both reef and mangrove environments, and often settles and burrows in areas with a mix of aerobic and anaerobic sediments (Hutchings et al., 1991; Palmer, 2010). Like other *Nereidids*, it is semelparous (only breeds once before dying) and often occurs in the vicinity of freshwater seeps where salinities are moderately reduced and variable (see Prevedelli, 1991; Zipperle and Reise, 2005). It is not unconditionally confined to its mucus-lined burrow during its life cycle and can survive for extended periods after being removed from its natural habitat (P.J. Palmer observations).

* Corresponding author.
E-mail address: Paul.Palmer@daf.qld.gov.au (P.J. Palmer).

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The polychaete-assisted sand filter (PASF) system was first described in 2010 as a means of removing nutrients from shrimp aquaculture pond wastewater, whilst at the same time providing the worms with a nutrient-rich feed source (Palmer, 2010). Unlike other passive sand filtration systems, PASF beds do not need to be back-flushed as polychaetes feeding at the sand surface help remove and digest micro algae, detritus and other organic matter that would otherwise saturate and prevent water penetration. PASF beds have been shown capable of removing up to 84% of suspended solids, 92% chlorophyll, 46% nitrogen and 63% phosphorus from shrimp pond wastewater, far exceeding the performance of current wastewater treatment practices using settlement ponds, as an example, and greatly reducing land areas needed for this (Campos et al., 2002; Palmer, 2010; Palmer et al., 2014, 2016, 2018).

Whilst only producing ~5000 t of farmed shrimp annually valued at $86 million AUD in 2016–17 (Mobsby, 2018), the industry in Australia supports many hundreds of jobs in rural communities and is projected to grow substantially (URL1). Most farms exist along the coast of Queensland and predominantly rear Black tiger shrimp (*Penaeus monodon*) as well as lower numbers of Banana shrimp (*Penaeus merguiensis*) and Kuruma shrimp (*Penaeus japonicus*). As the industry transitions from using wild to domesticated broodstock, feed ingredients known to promote broodstock fecundity are becoming increasingly important. The use of polychaetes including sandworms and bloodworms have proved critical for this purpose, with live worms being more effective as a dietary supplement than frozen worms (Meunpol et al., 2005, 2007; Palmer et al., 2014, 2018). With this demand for polychaetes, the PASF system is attractive for its potential to reliably supply consistent quality live worms at reasonable cost without impacting wild populations. However, if PASF beds are fed with wastewater from shrimp production ponds, there are risks of the worms ingesting material containing potential pathogens, such as white spot syndrome virus (WSSV) or infectious hypodermal and haematopoietic necrosis virus (IHHNV), and acting as a virus carrier if used either as bait by recreational anglers or as a dietary supplement to promote shrimp broodstock fecundity (Vijayan et al., 2005; Haditomo and Chilmawati, 2012; Desrina et al., 2013; Desrina, 2014; Haryadi et al., 2014).

Described here are TaqMan real-time qPCR data examining the potential for *P. helleri* reared in PASF beds to accumulate IHHNV from contaminated matter present in shrimp pond wastewater. The study focused on IHHNV, a commercially relevant small ssDNA virus also described as *Penaeus stylirostris* densovirus (*Pst*DNV) classified as type species of the *Penstyldensovirus* genus in the *Densovirinae* sub-family of the *Parvoviridae* (Saksmerprome et al., 2010; Rai et al., 2011; Tang and Lightner, 2002; Shike et al., 2000; King et al., 2012; Cotmore et al., 2014). The study used PASF beds having opportunistic access to wastewater from ponds used in a trial to determine the impact of high-load IHHNV infection on *P. monodon* growth performance and survival (Sellars et al., 2019). The qPCR data were used to investigate *P. helleri* as an IHHNV carrier, and remedial measures that could be used in commercial settings (e.g. short- and long-term purging treatments, and probiotics) to ameliorate the risks of PASF-worms transmitting IHHNV if used either as bait or as feed supplement for shrimp broodstock.

2. Materials and methods

2.1. Polychaete-assisted sand filter (PASF) bed design and operation

An experimental PASF system incorporating 10 × 54 m^2 sand beds was supplied with wastewater from 2 × 0.16 ha plastic-lined experimental ponds growing *P. monodon* (Fig. 1). Standardised bed management protocols were used (Palmer et al., 2016, 2018) except for the PASF wastewater being continually discharged rather than recirculated back to the ponds. PASF operations began on 28 Nov 2016 (Day 0), about 1 month (mo) after pond stocking with postlarvae as described below. Juvenile *P. helleri* (1-mo old) were stocked into beds on 29 and 30 Nov at ~2350 worms m^{-2}, and the worms grew at a rate consistent with previous trials using shrimp pond wastewater as a source of nutrients and organic matter.

Shrimp pond wastewater was distributed to each PASF bed in equal daily volumes (~400 L m^{-2} d^{-1}) and flow rates. The pattern and rate of water supply during normal dry-weather operation involved sun drying of the bed surface each afternoon between diurnal wastewater flows.

Shrimp Pond 1 wastewater was supplied to PASF Beds 1, 2, 5, 6, 9 and 10, and Shrimp Pond 2 wastewater was supplied to PASF Beds 3, 4, 7 and 8. Worms from Bed 3 were used in pilot TNA extractions leading to extraction method refinements (see Section 2.9) and were thereafter excluded from data analysis. From 9 Jan 2017 (PASF operational Day 42), PondToss™ probiotic (Keeton Industries) was applied to Beds 1, 5 and 9 by sprinkling the dry power (mixed homogenously with dry sand to afford a more even spread) over the bed surface each afternoon before the evening wastewater supply. In this novel application, PondToss™ was applied at 0.08 g m^{-2} d^{-1}, 4 times the rate recommended for use in aquaculture ponds (1 kg ha^{-1} every 5 days), for a 4 week period when amounts of IHHNV-contaminated matter in Pond 1 wastewater were expected to be high. This investigated the potential for probiotic application to reduce IHHNV loads in worms at a time when they might be harvested in commercial shrimp farming operations.

On 27 Feb 2017 (PASF operational Day 91), all PASF beds were transferred to clean unfiltered seawater supplies from recently dried and refilled ponds, with the same supply rates and patterns as used with the shrimp pond wastewater continued for 8 weeks (ie. till Day 147).

2.2. Shrimp ponds

Each of the 0.16 ha shrimp ponds was 1.5 m deep, completely lined with high-density polyethylene plastic, fitted with 2 paddle-wheels to provide water circulation and aeration, and contained 2.4 ML water. The ponds were maintained under simulated commercial grow-out conditions for *P. monodon* and were stocked with batches of post-larvae (PL15) derived from wild-captured female broodstock determined by TaqMan real-time qPCR analyses at various times pre- and post-spawning (Cowley et al., 2018) to be infected with IHHNV at varying severities (Sellars et al., 2019). Due to difference in IHHNV loads in their female parents, the shrimp cohort stocked into Pond 1 rapidly developed high-load IHHNV infections at 100% prevalence (Sellars et al., 2019). In contrast, the cohort stocked into Pond 2 only reached 100% infection prevalence toward the end of grow-out, and IHHNV infection loads remained low to moderate. Wastewater was pumped intermittently from the monk drain of each pond through 2 separate manifold systems that delivered measured rates of water to the different groups of PASF beds.

2.3. Wastewater percolation rates through PASF beds

Water percolation rates for all PASF beds were assessed on 8 Feb 2017, 2 days after PondToss™ probiotic application to Beds 1, 5 and 9 was terminated. This data was used as a proxy means of determining whether the probiotic had accelerated organic matter decomposition to potentially reduce the amounts of material containing IHHNV. Water percolation has previously been shown correlated with sand bed organic matter content (Palmer, 2010), and with relatively high organic particulate loads in the pond wastewater, higher percolation rates through PASF due to clearer pore spaces would have been supportive of accelerated decomposition. The maximum percolation rate possible for each bed was assessed volumetrically by applying a 60 cm head of water over the entire bed (as measured at the reticulated discharge point) and then by measuring the volume of water discharged over a 10 s period (extrapolated to L water min^{-1}).

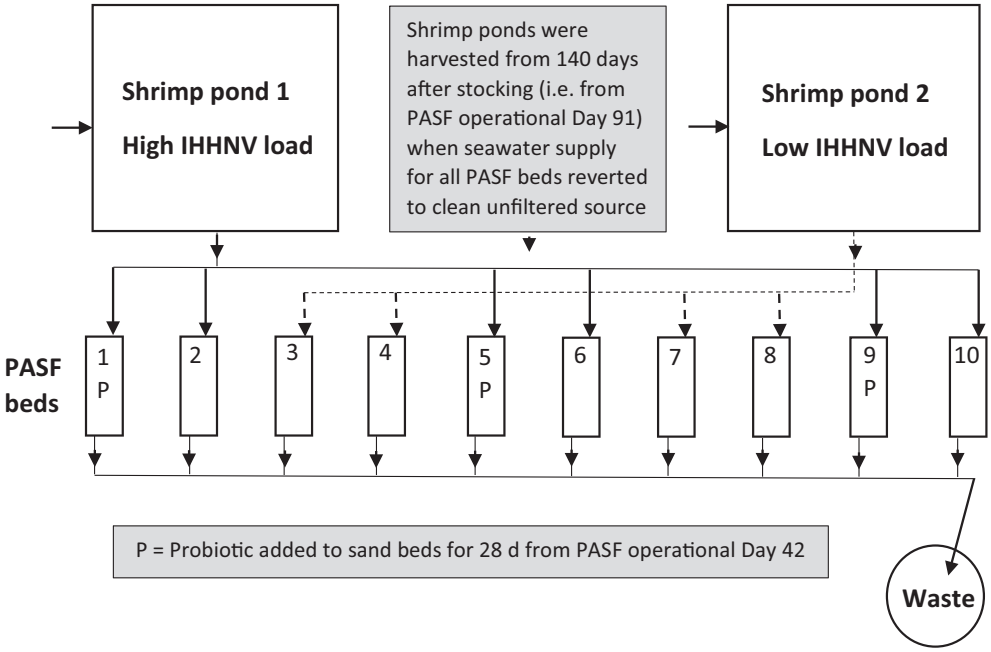

Fig. 1. Schematic diagram of experimental shrimp ponds and PASF beds showing treatments (not to scale; arrows show directional flow of seawater).

2.4. Worm sampling, cleansing, purging and preservation

Worms were sampled from selected beds on PASF operational Days 91 and 147 by using a shovel to over-turn sand in a small section of the bed. Irrespective of their size, the first 30 or more whole worms seen were collected into a bucket containing clean seawater. To remove mucus-laden sediment and debris, worms from each bucket were deposited onto damp sieves (4 mm square holes) placed over bins containing 30 L clean seawater to entice them to grovel their way through the sieve.

Worms were cleansed and purged in buckets containing high clarity seawater (10 μm sand-filtered, 34–35 ppt salinity, 26–27 °C, pH 8.0–8.1). To purge their gut contents, 15 cleansed worms from each PASF bed were maintained for 2 days without feeding in a 60 L bin containing gently-aerated, slow (1 L min^{-1}) flow-through seawater.

Cleansed only or cleansed and purged worms from the different beds and treatments were preserved in 5 mL RNAlater™ (Ambion) for 2 days at 4 °C before transfer to a − 20 °C freezer until thawed for processing.

2.5. Experimental design

Worms from all PASF beds that did not receive probiotics were sampled on 27 Feb 2017 (PASF operational Day 91) immediately before the supply of shrimp pond wastewater was changed to clean seawater. These samples provided a resource to compare IHHNV loads in cleansed only and cleansed and purged worms collected from either PASF Beds 2, 6 and 10 or Beds 4, 7 and 8 supplied wastewater from IHHNV high-load Pond 1 or IHHNV low-load Pond 2, respectively. On 24 Apr 2017 (PASF operational Day 147) when the PASF beds had received clean seawater for 8 weeks, 15 worms selected at random from each of Beds 2, 6 and 10 (to which no probiotic was applied), or Beds 1, 5 and 9 (to which probiotic was applied during the final 4 weeks these beds received wastewater from IHHNV high-load Pond 1), were again collected, cleansed and preserved in RNAlater. These samples provided a resource to compare IHHNV loads in cleansed worms from Beds 2, 6 and 10 sampled before and after being supplied clean seawater for 8 weeks, and the same for cleansed worms from Beds 1, 5 and 9 treated with the probiotics at the time IHHNV prevalence and loads in the *P. monodon* being reared in Pond 1 were high (Sellars et al., 2019). The 8 week supply of clean seawater also simulated how PASF beds could be operated in Australia with shrimp grow-out ponds harvested well before live worms would be needed in shrimp broodstock maturation diets.

2.6. Worm dissection

Preserved polychaetes were dissected to provide a standardised tissue type and mass for total nucleic acid (TNA) extraction. After thawing, worms were removed from RNAlater using sterile forceps and arranged on a clean cutting board parallel to a ruler to measure their length (mm), and then blotted briefly on absorbent paper towel to remove excess liquid. A sterile scalpel blade was then used to dissect 5 mm sections (i) immediately posterior to the worm head to avoid hard mouth parts that might compromise the extraction process or contain PCR inhibitory compounds and (ii) in the middle region of the body trunk, with somewhat longer sections of estimated similar mass dissected from (iii) the tip of the tail including the tail antennae and anus (Fig. 2).

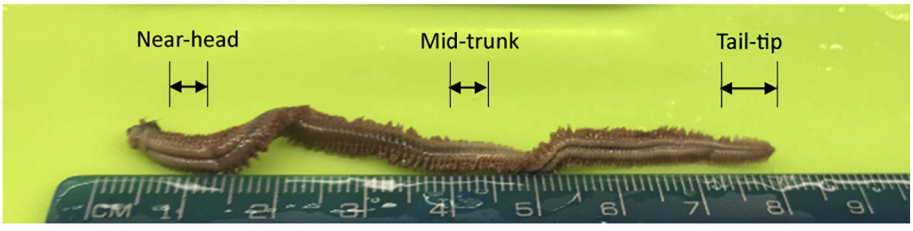

Fig. 2. Photograph of a preserved *Perinereis helleri* reared in a PASF bed identifying the near head, mid-trunk and tail-tip regions biopsied for TNA extraction and qPCR analysis.

2.7. Total nucleic acid (TNA = DNA + RNA) extraction

Biopsied tissue sections were transferred into wells of a 96-well deep-well plate sitting in ice. TNA was then extracted using the MagJET RNA kit (Thermo Scientific) using a protocol modified slightly from the instructions specified by the manufacturer, including omitting the DNase 1 digestion step (Sellars et al., 2019). Briefly, RLT buffer (QIAGEN) was added and the tissues disrupted by bead beating for 90 s in a Retsch MM300 TissueLyser (MEP Instruments). The plate was centrifuged at 3000 rpm for 5 min to pellet debris and collapse foaming. Lysate supernatant was then processed using a KingFisher Flex Magnetic Particle Processor and TNA was eluted from the magnetic beads in 80 μL RNase/DNase-free water. A 2 μL aliquot of the eluate was analysed using a Nanodrop ND8000 UV spectrophotometer (Thermo Scientific) to quantify TNA yields and purity. An aliquot of each TNA was normalized in a new plate to 50 ng μL^{-1} using RNase-free water, and plates of the stock and normalized TNA were stored at −80 °C.

2.8. Detection and quantification of IHHNV DNA by TaqMan real-time qPCR

IHHNV DNA in each normalized TNA sample was detected and quantified using the IHHNV q309 TaqMan real-time test as described previously (Cowley et al., 2018) except for amplifying a 20 μL reaction volume. Briefly, each PCR (25 μL) comprised 12.5 μL 2 x SensiFAST™ probe Lo-ROX mastermix (Bioline), 4 μL DNA (200 ng) template, 1.25 μL (0.9 μM) each primer IHHNV-q309F1 and IHHNV-q309R1, 1.25 μL (0.25 μM) TaqMan probe IHHNV-q309Pr1 and 4.75 μL water. A 20 μL aliquot of each PCR was amplified in a 384-well PCR plate using a Viia7 qPCR system (Applied Biosystems) employing 40 cycles of a standard thermal cycling profile (Cowley et al., 2018). Serial 10-fold dilutions of a synthetic linear IHHNV dsDNA template of calculated copy number between 800,000 and 0.8 copies per reaction were also analysed in each plate to generate a standard curve from which a cycle threshold (Ct) value could be converted to IHHNV ssDNA copies 200 ng^{-1} TNA using the QuantStudio v1.3 software (Thermo Fisher Scientific; Cowley et al., 2018).

2.9. Revised TNA extraction and qPCR methods

TNA yields in initial pilot tissue extractions varied markedly (3.8 to 403.0 ng μL^{-1}) and UV ($A_{260/280\ nm}$; $A_{260/230\ nm}$) spectral ratios identified the purity of many low-yield samples to be non-ideal. Ct values determined using the IHHNV qPCR test were also higher than ideal, with many approaching the test detection limit where technical accuracy becomes less reliable. TNA extractions repeated using approximately doubled tissue amounts combined with the water elution volume being reduced from 80 to 60 μL exacerbated extraction difficulties due to increased tissue lysate viscosity further compromising TNA yields and uniformity. To rectify this issue, an aliquot of each tissue lysate was diluted 6- to 8-fold with Lysis buffer (800 μL final volume) before extraction. This resulted in substantially improved TNA yields, purity and uniformity among the worm tissue extracts (46–184 ng μL^{-1}).

IHHNV qPCR data on near-head, mid-trunk and tail-tip regions of 30 worms tested in 2 pilot studies (to define robust analysis methods) identified no substantial differences in IHHNV DNA amounts among different sections of the same worm (Table 1). Based on this finding together with the improved TNA yields and purity obtained using diluted tissue lysate amounts, TNA extractions were standardised to use a weighed and reduced amount (40–60 mg) of tissue dissected from the region immediately posterior to the worm head.

2.10. Statistical analyses

For those worms in which IHHNV DNA was detected by qPCR, a geomean (geometric mean) of their IHHNV DNA load (IHHNV ssDNA copies 200 ng^{-1} TNA) was determined for each treatment group sampled at each time point examined. Data were analysed using a 2-part conditional generalised linear model (GLM; McCullagh and Nelder, 1989, MacNeil et al., 2009) in GenStat, 2018. Part 1 used a binomial GLM with the logit link function to determine the % positive worms, and Part 2 used a conditional gamma GLM with the log link to evaluate IHHNV loads limited to the qPCR-positive worms. Untransformed mean water percolation rates were compared using 1-way ANOVA.

Table 1
qPCR data on the presence and loads of IHHNV DNA in near-head, mid-trunk and tail-tip tissue sections including gastrointestinal track of groups of 18 or 12 worms sampled from PASF beds supplied wastewater from Ponds 1 or 2 rearing *P. monodon* with either high-load or low-load IHHNV infections, respectively.

Shrimp pond	PASF bed	Worm number	IHHNV qPCR geomean Ct value (20 μL)		
			Near-head	Mid-trunk	Tail-tip
IHHNV high-load Pond 1	1	1	35.3	36.5	33.9
		2	32.5	33.3	33.5
		3	38.2	39.3	UD
	2	4	35.0	33.5	36.1
		5	30.1	30.1	36.0
		6	31.6	31.9	29.2
	5	7	36.5	32.0	35.0
		8	30.1	32.3	36.3
		9	31.9	29.9	33.7
	6	10	37.4	38.4	39.2
		11	37.4	32.8	37.7
		12	31.3	35.7	34.0
	9	13	37.6	UD	UD
		14	36.9	UD	39.1
		15	35.7	36.2	30.4
	10	16	31.3	34.3	UD
		17	UD	35.2	38.5
		18	36.1	31.0	31.9
IHHNV low-load Pond 2	3	19	38.2	36.5	UD
		20	36.5	36.7	37.6
		21	38.4	38.2	UD
	4	22	UD	38.5	UD
		23	UD	UD	36.5
		24	UD	UD	UD
	7	25	UD	UD	UD
		26	UD	UD	UD
		27	UD	UD	UD
	8	28	UD	UD	UD
		29	UD	UD	UD
		30	UD	UD	UD

UD = undetermined.

3. Results

3.1. IHHNV loads in longitudinal worm sections

Perinereis helleri selected from PASF beds supplied wastewater from ponds rearing *P. monodon* identified to have either high-load IHHNV infections at high prevalence (Pond 1) or low-load IHHNV infections at more slowly-increasing prevalence (Pond 2) were tested to determine whether IHHNV DNA could be detected and if so, the uniformity at which loads were distributed between near-head, mid-trunk and tail-tip regions of the worm (Fig. 2, Table 1). Concentrations of TNA extracted from each sample were normalized and tested by qPCR, initially as 3 × 5 μL technical replicates (data not shown), and subsequently as a single 20 μL reaction to help improve data confidence and accuracy (Table 1). Ct values detected using either test method were generally well correlated considering that many were high (Ct range 29.2–39.2) and approaching the detection sensitivity limit of the qPCR test (Cowley et al., 2018). Among the 18 worms from PASF beds supplied

wastewater from the IHHNV high-load Pond 1, IHHNV DNA was detected in at least 2 of the 3 tissue sections from 17 (94%) and in 1 tissue section of the other worm. In contrast, IHHNV DNA was only detected in at least 2 of the 3 tissue sections from 3 of 12 (25%) and in 1 tissue section from 2 other worms from the beds supplied wastewater from the IHHNV low-load Pond 2 (Table 1). The qPCR Ct values for the various tissue regions of these 5 IHHNV-positive worms were also higher than those generally detected in most worms from the beds supplied wastewater from IHHNV high-load Pond 1. Moreover, no tissue region stood out to consistently possess the highest IHHNV DNA load (lowest Ct value) both within and between worms. Based on these data and the expectation that the near-head region would possess the most recently digested matter least likely to have undergone digestive breakdown, this worm region was selected for use in all subsequent qPCR analyses.

3.2. IHHNV loads and prevalence in Perinereis helleri

For *P. helleri* sampled on PASF operational Day 91, both the prevalence and loads of IHHNV DNA detected in worms reared in PASF beds supplied with wastewater from the IHHNV high-load Pond 1 were significantly higher ($P < .001$) than those detected in worms from beds supplied wastewater from the IHHNV low-load Pond 2. To show differences more clearly (Fig. 3, Table 2), Ct values were converted to IHHNV DNA copies 200 ng^{-1} TNA by fitting them to a standard curve generated for a 10-fold dilution series of synthetic linear IHHNV dsDNA of accurately defined copy number. In the 3 PASF beds (2, 6, 10) supplied wastewater from Pond 1, IHHNV DNA was detected in 35 of 36 (97%) worms at loads ranging from 0.8 to 878 IHHNV DNA copies 200 ng^{-1} TNA (geomean for each bed = 45 to 66 IHHNV DNA copies 200 ng^{-1} TNA). In the 3 PASF beds (4, 7, 8) supplied wastewater from Pond 2, IHHNV DNA was only detected in 5 of 36 (14%) worms at low (< 3 IHHNV DNA copies 200 ng^{-1} TNA) loads nearing the detection sensitivity limits of the qPCR test.

After being purged of their gut contents for 2 days in clean seawater, IHHNV DNA remained detectable in most worms (42 of 45 = 93%) collected from PASF Beds 2, 6 and 10 but at much reduced loads ranging from 2 to 213 IHHNV DNA copies 200 ng^{-1} TNA (geomean for each bed = 10 to 23 IHHNV DNA copies 200 ng^{-1} TNA; Fig. 3, Table 2). The very low levels of IHHNV DNA detected in worms collected from PASF Beds 4, 7 and 8, remained at similarly low levels (≤ 2 IHHNV DNA copies 200 ng^{-1} TNA) after purging in those few worms (5 of 45 = 11%) in which IHHNV DNA remained detectable.

Operating the PASF beds for a further 8 weeks with clean seawater (PASF operational Days 91 to 147) significantly reduced prevalence ($P < .05$) and loads ($P < .001$) of IHHNV DNA detected by qPCR (Fig. 4, Table 3). Indeed, IHHNV DNA was only detected in 20 of the 45 (44%) worms tested after this period at extremely low loads (< 4 IHHNV DNA copies 200 ng^{-1} TNA) nearing the detection sensitivity limit of the qPCR test.

On PASF operational Day 147, worms were also sampled from the PASF Beds (1, 5, and 9) treated daily with the commercial PondToss™ probiotic over the final 4 week period they were supplied wastewater from IHHNV high-load Pond 1. In some of these worms, qPCR testing again detected low loads of IHHNV DNA nearing the detection sensitivity limit of the qPCR test. No significant differences were found between the mean percentages of positive detections ($P = .708$) or

Table 2
Mean percentages of *Perinereis helleri* in which IHHNV DNA was detected by qPCR and geomeans of IHHNV DNA copies 200 ng^{-1} TNA detected among those testing qPCR-positive in each of the 3 replicate PASF beds supplied with wastewater from either IHHNV high-load shrimp Pond 1 (Beds 2, 6, 10) or IHHNV low-load shrimp Pond 2 (Beds 4, 7, 8) after being cleansed only (not purged) or cleansed and purged of their gut contents in clean seawater for 2 days.

Shrimp pond IHHNV load	PASF bed		IHHNV positive	IHHNV DNA copies 200 ng^{-1} TNA*
	No.	Treatment	Mean % ± S.E.	Geomean ± S.E.
High	2, 6, 10	Not purged	97 ± 4 [a]	57.4 ± 9.1 [a]
		Purged	93 ± 6 [a]	16.4 ± 2.6 [b]
Low	4, 7, 8	Not purged	14 ± 9 [b]	1.4 ± 0.2 [c]
		Purged	11 ± 7 [b]	1.4 ± 0.3 [c]

S.E. = standard error.
Means or geomeans followed by different letters are significantly ($P < .05$) different.
* Analysis conditional on IHHNV being detected in a worm (i.e. IHHNV qPCR positives only).

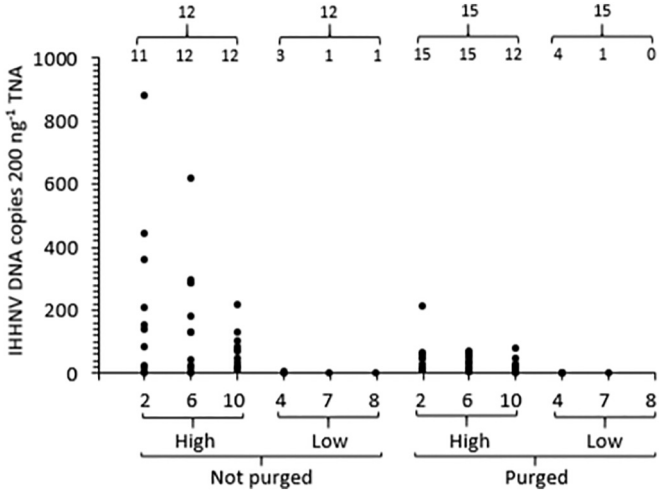

Fig. 3. qPCR data on the prevalence and loads of IHHNV DNA (IHHNV DNA copies 200 ng^{-1} TNA) in *Perinereis helleri* sampled from PASF beds supplied with wastewater from either IHHNV high-load shrimp Pond 1 (Beds 2, 6, 10) or IHHNV low-load shrimp Pond 2 (Beds 4, 7, 8). Data on each PASF bed are shown for worms either cleansed only (not purged) or cleansed and purged of their gut contents in clean seawater for 2 days. The numbers of worms tested from each PASF bed (n = 12 or 15) and in which IHHNV DNA was detected are shown.

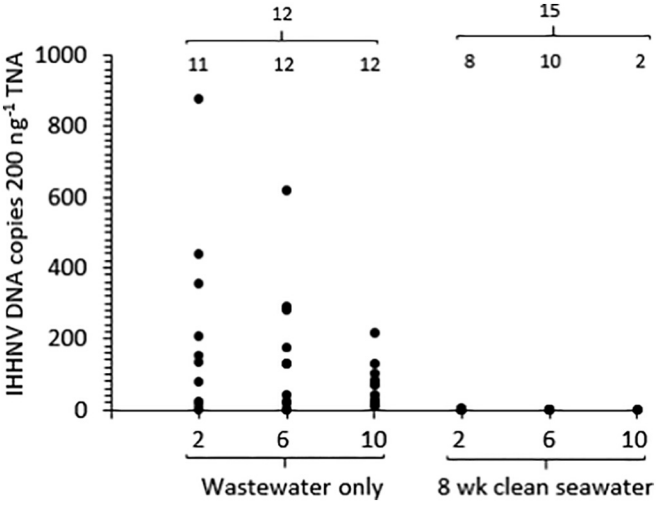

Fig. 4. qPCR data on the prevalence and loads of IHHNV DNA (IHHNV DNA copies 200 ng^{-1} TNA) in *Perinereis helleri* sampled from PASF Beds 2, 6 and 10 supplied with wastewater from IHHNV high-load shrimp Pond 1. Data on each PASF bed are shown immediately before (wastewater only) and after the PASF beds were supplied with clean seawater for 8 weeks. The numbers of worms tested from each PASF bed (n = 12 or 15) and in which IHHNV DNA was detected are shown.

Table 3
Mean percentages of *Perinereis helleri* in which IHHNV DNA was detected by qPCR and geomeans of IHHNV DNA copies 200 ng^{-1} TNA detected among those testing qPCR-positive in each of the 3 replicate PASF beds supplied with wastewater from IHHNV high-load shrimp Pond 1 (Beds 2, 6, 10) and sampled either immediately before (none) or after being supplied with clean seawater for 8 weeks.

Shrimp pond IHHNV load	PASF bed		IHHNV positive	IHHNV DNA copies 200 ng^{-1} TNA*
	No.	Treatment	Mean % ± S.E.	Geomean ± S.E.
High	2, 6, 10	None	97 ± 5 [a]	57.4 ± 5.5 [a]
		Clean seawater	44 ± 13 [b]	1.6 ± 0.2 [b]

S.E. = standard error.
Means or geomeans followed by different letters are significantly ($P < .05$) different.
* Analysis conditional on IHHNV being detected in a worm (i.e. IHHNV qPCR positives only).

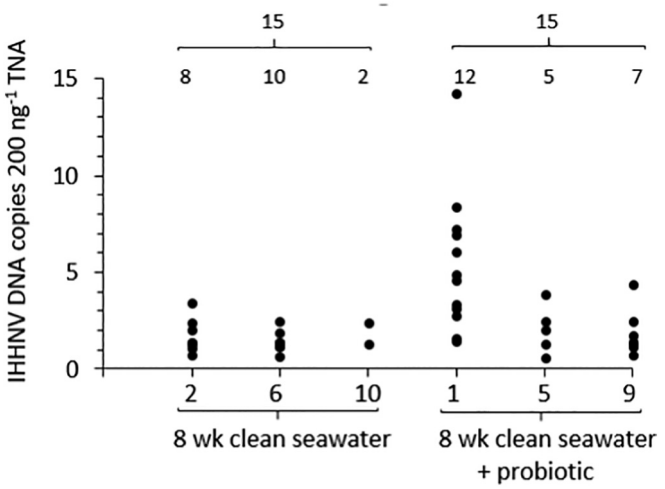

Fig. 5. qPCR data on the prevalence and loads of IHHNV DNA (IHHNV DNA copies 200 ng^{-1} TNA) in *Perinereis helleri* sampled from PASF Beds 2, 6 and 10 and Beds 1, 5 and 9 supplied clean seawater for 8 weeks after being supplied wastewater from IHHNV high-load shrimp Pond 1. For PASF Beds 1, 5 and 9, PondToss™ probiotic was applied daily over the final 4-week period they were supplied with shrimp pond wastewater. The numbers of worms tested from each PASF bed ($n = 15$) in which IHHNV DNA was detected are shown.

Table 4
Mean percentages of *Perinereis helleri* in which IHHNV DNA was detected by qPCR and geomeans of IHHNV DNA copies 200 ng^{-1} TNA detected among those testing qPCR-positive in each of the 3 replicate PASF beds supplied with wastewater from IHHNV high-load shrimp Pond 1 after being supplied with clean seawater for 8 weeks either without (Beds 2, 6, 10) or with the application of PondToss™ probiotic (Beds 1, 5, 9) as described in Materials and methods.

Shrimp pond IHHNV load	PASF bed		IHHNV positive	IHHNV DNA copies 200 ng^{-1} TNA*
	No.	Treatment	Mean % ± S.E.	Geomean ± S.E.
High	2, 6, 10	None	44 ± 16 [a]	1.6 ± 0.4 [a]
	1, 5, 9	PondToss™	53 ± 16 [a]	2.6 ± 0.6 [a]

S.E. = standard error.
Means or geomeans followed by different letters are significantly ($P < .05$) different.
* Analysis conditional on IHHNV being detected in a worm (i.e. IHHNV qPCR positives only).

IHHNV DNA loads ($P = .206$) in worms sampled on the same day from the PASF beds with (1, 5 and 9) and without (2, 6 and 10) probiotic applications (Fig. 5, Table 4).

The water percolation rates through these beds were also not affected significantly ($P = .60$) by probiotic addition to the sand bed surface. Using a 60 cm head, the average (± S.E., $n = 3$ or 4) water discharge rate from the probiotic-treated PASF beds (1, 5 and 9) was 87.0 ± 2.7 L min^{-1} and similar to that of Beds 2, 6 and 10 (90.2 ± 6.6 L min^{-1}) supplied wastewater from the same pond (Pond 1) without the probiotic treatment and Beds 3, 4, 7, 8 (92.6 ± 1.5 L min^{-1}) supplied wastewater from IHHNV low-load Pond 2.

4. Discussion

Here we examined the capacity of sand worms (*Perinereis helleri*) to accumulate IHHNV from organic matter discharged into PASF beds flooded twice daily with wastewater from simulated aquaculture ponds rearing *Penaeus monodon* with differing IHHNV prevalence and infection loads (Sellars et al., 2019). IHHNV DNA was detected unequivocally by real-time qPCR in tissue sections containing intestinal tract in most (35 out of 36) *P. helleri* sampled from 3 PASF beds supplied with wastewater from IHHNV high-load Pond 1. In contrast, it was only detected at marginal loads approaching the detection sensitivity limit of the IHHNV qPCR test in a few (5 of 36) worms tested from 3 beds supplied wastewater from Pond 2 rearing *P. monodon* with a lower prevalence of much ($> 10^4$-fold) lower-load IHHNV infections (Sellars et al., 2019). For the PASF beds supplied wastewater from the IHHNV high-load Pond 1, worms purged of their gut contents by maintaining them in clean seawater for 2 days possessed markedly reduced IHHNV DNA amounts. Even more pronounced reductions in IHHNV DNA prevalence and loads were found in worms from PASF beds supplied with clean seawater for 8 weeks before harvest.

There has been some success in domesticating and selectively breeding *P. monodon* broodstock in Queensland (Preston et al., 2009, 2010; Norman-López et al., 2015), but the emergence of white spot disease (WSD) in the Logan River aquaculture region in Dec 2016 (Oakey and Smith, 2018) resulted in the most advanced breeding populations being destroyed. Due partly to this and projected expansions in the farming of this species in Australia, concerns are emerging about security in the supply of wild broodstock and their potential biosecurity risk. While driving new vigour in larger farms to establish new breeding lines of *P. monodon*, enthusiasm is being tempering by the known difficulties in breeding this species in captivity (Preston et al., 2009, 2010). Pivotal to these difficulties has been the poor fecundity of early generations of captive-reared *P. monodon* broodstock believed to be caused by non-natural rearing environment and dietary factors. To remediate dietary deficiencies, the protein and lipid profiles of fresh feeds such as molluscs and squid, and particularly live feeds such as annelid worms (polychaetes), have been considered essential for maturing broodstock gonads (Meunpol et al., 2005, Coman et al., 2007, see review Chimsung, 2014). Whilst no ideal nutrient profile has yet been determined for *P. monodon*, various *Perinereis* species contain levels of progesterone (E_2) and 17-alpha hydroxyprogesterone which are important in shrimp oocyte maturation (Meunpol et al., 2007, 2010). This supports their dietary use without treatments such as freezing or drying that may compromise such endocrine stimulants.

However, despite their dietary benefits, there are well-recognised downsides to feeding fresh and live feed supplements such as polychaetes to shrimp broodstock. Important among these, and particularly so for shrimp breeding lines selected to be specific pathogen free (SPF), is their risk of carrying and transmitting potential pathogens (Vijayan et al., 2005; Haditomo and Chilmawati, 2012; Desrina et al., 2013; Desrina, 2014; Haryadi et al., 2014; Chimsung, 2014). The detection of IHHNV DNA in *P. helleri* reared in the PASF system highlight this downside, and shrimp challenge trials are needed to assess the extent to

which IHHNV remains infectious as it transitions through the *P. helleri* digestive tract. With respect to ameliorating virus transmission risks, purging worms of their gut contents by exposing them to clean seawater for 2 days markedly reduced IHHNV DNA loads. Maintaining PASF beds with clean seawater for 8 weeks after ceasing the supply of the shrimp pond wastewater laden with organic matter resulted in even less IHHNV DNA being detected. Whilst not mitigating the potential for pathogen transmission altogether as in growing SPF polychaetes in biosecure systems (Poltana et al., 2007), these data suggest that IHHNV does not infect and amplify in *P. helleri*. While such purged polychaetes might be adequate as a maturation diet supplement for shrimp broodstock with known IHHNV infections, more stringent implementations of these virus clearing processes would need to be evaluated for their ability to unconditionally mitigate any risks of them transmitting infection.

In support of polychaetes like *P. helleri* being only a passive carrier of crustacean-specific viruses, the related polychaete species *Perinereis nuntia* suffered no ill effects from ingesting shrimp tissue containing high loads of infectious WSSV (Laoaroon et al., 2005). Even though WSSV DNA was clearly detected in the *P. nuntia* by PCR, *P. monodon* fed upon them showed no evidence of infection. A similar loss of WSSV infectivity over a short time period has also been noted in another related species *Perinereis cultrifera* examined at a time they tested PCR-positive for WSSV DNA after ingesting WSSV-contaminated tissue (Shalini et al., 2016). However, whether the inability of these polychaetes to transmit WSSV was due to virus loads being too low to establish a productive infection or to virus infectivity being compromised by worm digestive tract factors was not established.

While these data on WSSV and the data presented here on IHHNV suggest that polychaetes are passive virus carriers with potential to compromise virus infectivity, it has been suggested that wild *P. cultrifera* should be held for up to a week in clean seawater to ensure that their digestive tract contents have been purged completely before using them in shrimp maturation diets (Shalini et al., 2016). For PASF-cultured *P. helleri*, there is potential to extend gut purging over months, if desired, using clean seawater combined with diets based on sterile fish meal (Palmer et al., 2014). Due to the high value of live polychaetes as either bait or a shrimp broodstock feed supplement, and the high numbers of worms that can be reared using the PASF system (Palmer et al., 2016), any additional impost and expense of extended gut purging would be inconsequential.

Perinereis helleri is well suited to rearing in PASF systems supplied with shrimp pond wastewater. This is due to the species' tolerance of a wide variety of sediments and substrates loaded with decomposing organic matter in its natural tropical and subtropical habitats as well as moderate salinity variations in land-based sand beds impacted intermittently by rainfall. Its indiscriminate feeding preferences are also effective in degrading plant, animal or bacterial biomass that would otherwise rapidly clog the sand bed matrix, and thus aid in reducing loads of pathogens existing in this biomass. The *P. helleri* reared in the PASF beds reported here maintained their expected operating parameters irrespective of many accumulating IHHNV. Less expected, however, was qPCR data indicating the presence of IHHNV DNA, sometimes in similar amounts to the near-head region containing newly-ingested, semi-solid, and thus less digested biota, in mid-trunk and tail sections of worms where the digestive tract contents had liquified. This suggested that IHHNV particles might tolerate the *P. helleri* digestive tract environment. Alternatively, as the IHHNV qPCR amplifies only a 98 nt fragment of the IHHNV DNA genome (Cowley et al., 2018), it can potentially detect semi-degraded DNA with similar efficiency to non-degraded genomic DNA protected within virus particles. Shrimp challenge trials are thus needed to assess the potential infectivity of IHHNV detected by qPCR in fore and aft regions of the *P. helleri* digestive tract.

The PondToss™ probiotic is held to contain 2 billion cfu g^{-1} of mainly undefined naturally-occurring microbes, to promote organic solid and sludge decomposition and to reduce nitrogenous compounds in shrimp or fish ponds. Probiotics targeted to aquaculture systems have also been suggested to have antiviral activities possibly mediated by non-specific binding of virus particles restricting their uptake by susceptible hosts, by providing micronutrients essential to host robustness, and by non-specifically stimulating host defence mechanisms (Moriarty et al., 2005; Balcazar et al., 2007). Despite these claims, no evidence was obtained for a 4 week application of PondToss™ probiotic further reducing IHHNV DNA loads detected in *P. helleri* sampled from PASF beds subsequently supplied clean seawater for 8 weeks or increasing water percolation rates through the PASF beds. The potential reasons for this are many and varied and would require more detailed and targeted investigations to determine.

Taken together, the data presented here indicate that IHHNV-containing matter discharged in shrimp pond wastewater can passively accumulate at low levels in *Perinereis helleri* reared in PASF beds. They also show IHHNV to be rapidly discharged from worms following exposure to clean seawater for 2 days to purge their gut contents. Whilst promising, shrimp challenge trials will be needed to determine stringencies for the gut purging process needed to provide a fail-safe means of producing virus-free worms. Until then, sand beds rearing SPF *P. helleri* on sterile diets or nutrient-rich water from biosecure pond/tank systems producing either SPF shrimp broodstock (Palmer et al., 2016) or biofloc-based feed supplements such as Novacq™ (Anand et al., 2014; Glencross et al., 2014), as examples, would be expected to ameliorate virus-transmission risks and expedite the use of live polychaetes as a dietary supplement to promote fecundity in captive-reared *P. monodon* broodstock.

Organisational contributions

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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