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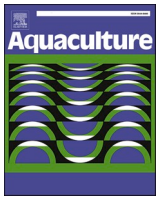

Passive eDNA sampling to monitor infectious hypodermal and haematopoietic necrosis virus (IHHNV) in pond-reared giant black tiger shrimp (*Penaeus monodon*) aquaculture systems

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ABSTRACT

Passive sampling of environmental nucleic acids (eDNA and eRNA) has potential as a simple, effective, and cost-efficient method for pathogen surveillance in aquaculture. This study evaluated passive eDNA sampling for targeted detection and quantification of infectious hypodermal and haematopoietic necrosis virus (IHHNV) in giant black tiger shrimp, *Penaeus monodon,* aquaculture ponds. The ability of eDNA to estimate IHHNV host infection was evaluated through two investigations, comparing TaqMan qPCR detections of IHHNV from passively sampled eDNA and shrimp tissue samples from the same ponds. Passive sampling of eDNA involved timed submersion of membrane filters, within protective housings, in shrimp ponds. The effect of submersion duration on IHHNV detection was initially evaluated by submerging passive samplers for 1, 2, 4, 6 and 24 h across five ponds at 159-169 days of shrimp culture (DOC). Longer passive sampler submersion durations increased IHHNV detection from eDNA, and IHHNV loads were strongly correlated between eDNA and shrimp tissue. Passive eDNA samplers were then deployed at a farm-scale in 22 earthen shrimp ponds (125-129 DOC) for 24 h, given the higher IHHNV detection at 24 h in the initial study. The farm-scale deployment showed 95 % agreement in identifying ponds as IHHNV positive using eDNA and shrimp samples. Mean IHHNV loads in ponds were strongly correlated between eDNA and shrimp tissue (R^2 = 0.95), indicating the potential of eDNA detection to approximate host infection levels. These findings provide evidence to advance the use of passive eDNA sampling for accurate and efficient monitoring of IHHNV in shrimp aquaculture systems.

1. Introduction

Infectious disease is a significant challenge to the ecological and economic sustainability of global shrimp aquaculture (FAO, 2022; Stentiford et al., 2012). Timely, accurate and cost-effective pathogen detection is critical for effective shrimp disease mitigation and management (Major et al., 2023). Molecular technologies, primarily qPCR, form the basis of current pathogen detection and disease diagnosis in shrimp aquaculture (Kumar et al., 2021; WOAH, 2023a). Current practices for shrimp pathogen detection rely on the collection and analysis of host tissue samples. Although host tissue sampling remains a requisite of disease diagnosis (i.e. for confirmation of a pathogenic agent in clinically affected or apparently healthy animals) (WOAH, 2023a), tissue collection can present challenges for non-diagnostic purposes, such as for surveillance and monitoring. Specifically, pathogen surveillance programs can rapidly become prohibitively expensive due to the costs and requirements of extensive host tissue sampling and analysis (Huerlimann et al., 2020; Reich et al., 2018). For example, shrimp production ponds may be stocked at densities ranging between 20 and 60 shrimp m^{-2} (Eid et al., 2020; Mena-Herrera et al., 2006), translating to a theoretical pond (~0.5 - 1 ha) population of 100,000–600,000 shrimp (e.g. Zeng et al., 2020), with farms often comprising tens to

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hundreds of ponds. To conduct a farm-scale pathogen monitoring program aiming to detect a target pathogen with a prevalence of 10 % within a large pond population, at least 39 shrimp per pond must be sampled, assuming reasonable test sensitivity (0.8 for pooled tissue samples of three individuals; epitools.ausvet.com.au). Multiple tissue types per animal may also be required for the detection of multiple pathogen targets (WOAH, 2023a). For a monitoring program to provide early detection capability, and valuable spatiotemporal insights to improve pathogen management, the tissue sampling effort must be scaled for the number of ponds on the farm and repeated at regular intervals. Although recognised as best practice, even with pooling of tissue samples to reduce analysis costs, sample numbers and sampling effort rapidly become cost and time-prohibitive (Brunner, 2020) and often prevent the adoption of farm-scale pathogen analysis and monitoring programs.

1.1. Environmental sampling for pathogen surveillance

The collection and analysis of genetic material obtained from environmental samples, termed environmental DNA (eDNA) or RNA (eRNA), collectively termed eNA, is proposed as an alternative to tissue sampling for monitoring of pathogen presence in aquatic systems (Bass et al., 2023; WOAH, 2022) and is rapidly gaining popularity for effective application to aquaculture (Bastos Gomes et al., 2017; Benedicenti et al., 2024; Duan et al., 2025; Ip et al., 2024; Oladi et al., 2024; Rusch, 2021; Song et al., 2025; Wang et al., 2022). Existing in various physical states, eNA may be derived from entire organisms embedded within biofilms to extracellular nucleic acid, including all intermediate variations (Power et al., 2023). In the context of pathogen discovery, surveillance and monitoring, eNA is defined as nucleic acids of pathogenic agents extracted from 'true' environmental samples, such as water, soil, sediment or biofilm (WOAH, 2022). Positive molecular detection from eNA samples cannot confirm host infection, as positive detection from the environment does not demonstrate the infection of a susceptible host (WOAH, 2022). However, detections from eNA samples may serve as a proxy to indicate infection of host animals within a system (Bass et al., 2023). As a proxy, eNA samples are a valuable tool for pathogen surveillance and monitoring, and provide considerable advantages over sampling of host tissue for this purpose (WOAH, 2022). In contrast to individual tissue sampling, the number of eNA samples required to detect a pathogen decreases relative to an increasing population size (given a fixed pathogen prevalence), as more infected individuals contribute to shedding eNA into the system (Brunner, 2020). As such, eNA allows broader spatial and temporal coverage, increased specificity, sensitivity, and efficiency of surveillance efforts, as a small number of eNA samples can represent multiple host animals, or whole populations (Bass et al., 2023; Brunner, 2020; Hall et al., 2018). The capacity for a single eNA sample to simultaneously capture multiple pathogen targets further eliminates the requirement to sample different target tissues, greatly reducing sampling requirements and enabling more comprehensive analyses (Benedicenti et al., 2024; Sieber et al., 2024). Given the improved efficiency, eNA sampling can be applied routinely throughout the production cycle to monitor pathogen presence in the absence of overt clinical signs, enabling improved scope for early detection and proactive disease management (Farrell et al., 2021). Additionally, eNA sampling avoids the requisite of handling, stressing or sacrificing host animals during monitoring (Bowers et al., 2021) which is particularly advantageous for monitoring high-value aquaculture populations such as broodstock, or in biosecurity hazard points such as intake water canals, where the host species (e.g. shrimp) are not cultured (WOAH, 2022).

Given its significant benefits, eNA monitoring is increasing in popularity and has been applied across diverse environmental systems for targeted pathogen and parasite detection (e.g., reviewed in Bass et al., 2023). Despite its benefits for these applications, conventional eNA sampling methods can be challenging due to the need for active filtering, concentration, or storage of large environmental sample volumes (Tsuji et al., 2019; Verdier et al., 2022; Wang et al., 2022). Active filtering methods require specialised equipment, such as peristaltic pumps or in-line filters which are often difficult to operate, time and cost inefficient, easily contaminated, and result in an inability (or reluctance) from non-experts to conduct field sampling (Bessey et al., 2022; Chen et al., 2022; Rusch, 2021; Villacorta-Rath and Burrows, 2021; Zaiko et al., 2018). Sequential water collection and filtration across a farm may also represent a risk of cross-contamination across multiple sample sites leading to false positives, or the translocation of viable pathogens across a farm system if insufficient decontamination and sterilisation of equipment occurs. Active filtration methods also face challenges with standardising sampling; for example, filtering the same sample volume from clear versus turbid water may require a disparate number of filter membranes or may be infeasible if filters become clogged by high particulate levels (Bessey et al., 2022; Chen et al., 2022; Goldberg et al., 2016; Huerlimann et al., 2020; Ip et al., 2024; Wittwer et al., 2018). Difficulties in standardisation of sampling can affect downstream processing and analysis and reduce the ability to make relative comparisons between sample sites.

1.2. Passive environmental sampling

An emerging alternative, termed 'passive eNA sampling', is gaining popularity as a viable method with comparable performance to active eNA collection (Bessey et al., 2021, 2022; Kirtane et al., 2020; Rafiee et al., 2021; Verdier et al., 2022; Vincent-Hubert et al., 2017). Passive eNA sampling involves incubation of a filter medium within the environmental matrix to enable passive settlement or binding of genetic material to the filter (Bass et al., 2023; Bessey et al., 2022). The filter medium is retrieved, placed directly into a preservative solution, and processed for nucleic acid extraction and further analysis. Passive eNA sampling is considerably more time- and cost-efficient than active collection methods, requires a lower level of expertise to operate, and poses fewer risks of cross-contamination of equipment (Chen et al., 2022). The simplicity of passive eNA sampling makes it well-suited for large-scale, long term monitoring efforts, and remote deployment by non-experts (Bessey et al., 2022; Mejías-Molina et al., 2024a).

Passive sampling for environmental pathogen surveillance has a well-established history, and was first introduced by Moore in 1948 for the detection and tracing of *Salmonella* in sewage using folded gauze swabs submerged for 48 h (Moore, 1948). The application of passive sampling has seen recent renewal in a diverse range of studies, including for (waste)water-based epidemiology (WBE) of human pathogens and aquatic systems (Bivins et al., 2022; Hayes et al., 2022; Hayes and Gagnon, 2024; Jones et al., 2022; Mejías-Molina et al., 2024b; Mejías-Molina et al., 2024a; Mejías-Molina et al., 2023; Rafiee et al., 2021; Schang et al., 2021; Shakallis et al., 2022; Sikorski and Levine, 2020; Vincent-Hubert et al., 2022) (Table 1). The wide application to water-based epidemiology studies highlights the promise of passive eNA collection for pathogen monitoring in aquaculture.

1.3. Infectious hypodermal and haematopoietic necrosis virus

Infectious hypodermal and haematopoietic necrosis virus (IHHNV, *syn. Penaeus stylirostris* penstyldensovirus 1; PstDV1) is a nonenveloped, single-stranded DNA virus in the family *Parvoviridae* (ICTV, 2025). IHHNV was listed in the first edition of the World Organisation for Animal Health (WOAH) Aquatic Code and is the only WOAH listed pathogen that is frequently detected in the Australian shrimp aquaculture industry (Arbon et al., 2022; OIE, 1995; WOAH, 2023b). While IHHNV has been associated with high mortality in *P. stylirostris* (Bell and Lightner, 1984; Lightner et al., 1983), infections in giant black tiger shrimp, *P. monodon,* are typically subclinical, though chronic infections have been associated with reduced growth rates and deformities (Dhar et al., 2014; Flegel, 2006). IHHNV infections are considered systemic,

Table 1
Examples of aquatic field-deployed passive samplers for the detection of environmental nucleic acids.

Filter material	Submersion time	Target	Field environment	Filter material housing	Reference
Montmorillonite clay * Granular activated carbon**	7 and 21 d	*Lampsilis siliquoidea* (mussel) eDNA and universal fish mitochondrial 12S rRNA gene	Freshwater creek	* Two 40 μm cell strainers ** Polyester mesh bag All materials housed within a 3" dia. x 16" PVC pipe with water flow holes	(Kirtane et al., 2020)
Gauze (Moore swab)	16 h	SARS-CoV-2 eRNA	Human wastewater	Stainless steel wire sieve	(Rafiee et al., 2021)
Gauze (Moore swab) Cellulose nitrate filter membrane (0.45 μm) Cotton buds	3-7 and 24 h	SARS-CoV-2 eRNA	Human wastewater	3D printed 'torpedo'	(Schang et al., 2021)
3D-printed hydroxyapatite	24 h	*Asellus aquaticus* (isopod) eDNA	Freshwater pond	No housing	(Verdier et al., 2022)
Cellulose ester filter membrane (CEFM) Electropositive nylon filter membrane	4, 8, 12, 24 h	Universal fish mitochondrial 16S rDNA eDNA	Open ocean (tropical and temperate)	All materials housed within a pearl oyster aquaculture frame	(Bessey et al., 2021)
CEFM CEFM with 1 % chitosan or 3 % chitosan CEFM overlayed with electrospun nanofibers and 1 % chitosan Cotton fibres *** Hemp fibres *** Sponge with embedded zeolite or activated carbon	5 min, 10 min, 30 min, 1 h and 18 h	Universal fish mitochondrial 16S rDNA eDNA	3 million L marine mesocosm (Main tank; The Aquarium of Western Australia [AQWA])	*** Nylon bag All materials housed within a pearl oyster aquaculture frame	(Bessey et al., 2022)
Glass fibre filter membrane	0.5, 8, 24, and 72 h	Universal fish mitochondrial 12S rRNA gene	Freshwater lake (~2 ha)	Polyethylene terephthalate teabags	(Chen et al., 2022)

yielding consistent genetic detection from tissues of ectodermal, mesodermal, endodermal, and enteric origin (Arbon et al., 2024; Chayaburakul et al., 2005; Hou et al., 2023; Hsieh et al., 2006; Lee et al., 2021). IHHNV replicates in the nucleus of host cells, with viral material released into the surrounding environment via cell lysis or shedding of infected cells (Chayaburakul et al., 2013; Dominic et al., 2023).

Shrimp aquaculture ponds are discrete aquatic systems housing dense monocultures, where mechanical aeration is used to reduce physicochemical stratification of the water column and, where possible, water quality is actively maintained within optimal ranges. Pond environments are expected to provide favourable conditions for eNA accumulation, and thus, it is hypothesised that eNA-based pathogen monitoring would be effective in these settings. This study assessed passive eDNA sampling for determining viral pathogen presence in *P. monodon* aquaculture ponds, using targeted detection of IHHNV as a model pathogen. Specifically, we investigated a) the effect of passive eDNA sampler submersion duration on IHHNV detection, and b) the potential of passively sampled eDNA as a proxy for tissue samples in quantifying IHHNV load in shrimp.

2. Methods

2.1. Experimental design

This study was conducted at a commercial *P. monodon* grow-out farm in North Queensland, Australia. Shrimp samples and passively sampled eDNA were collected from earthen production ponds (0.8-1 ha) within the 2022-2023 production season during the period of 125 to 169 days of shrimp culture (DOC). Throughout the sampling period, there were no observed clinical signs of disease. Water quality parameters, including temperature (°C), pH and dissolved oxygen (DO; ppm) were recorded for each pond on the day of sampling. Salinity was also measured from each pond within a week of sampling.

2.2. Submersion duration study

Initially, the effect of passive eDNA sampler submersion duration on the detection of IHHNV was assessed across five shrimp production ponds. In each of the ponds, 10 passive eDNA samplers (two replicates per time point) were deployed at a consistent location. Passive samplers were secured from pond inspection jetties, approximately 30 cm below the water surface and approximately 2 m from the pond edge. The inspection jetties were in approximately the same position in each pond and, among other tasks, served as the feed tray inspection point. Duplicate eDNA samplers were retrieved from each pond after 1, 2, 4, 6 and 24 h of submersion. *P. monodon* ($n = 10$) were also sampled from each pond during the same period of eDNA sampler deployment. A total of 10 shrimp were collected from each pond, as the ponds were known to be positive for IHHNV at high prevalence based on previous qPCR analysis (data not shown). The number of shrimp sampled from each pond enabled theoretical detection of ~30 % target prevalence from each pond population, assuming a test sensitivity of 0.9 from individual tissue samples (epitools.ausvet.com.au). From each individual shrimp, pleopod, gill and hepatopancreas tissue was sampled (Table 2).

2.3. Farm-scale monitoring

To evaluate the potential of passively sampled eDNA for proxy quantification of pathogen load in shrimp hosts, eDNA sampling was conducted in parallel to shrimp tissue sampling from a total of 22 commercial ponds. Based on results from the submersion duration study, duplicate passive eDNA samplers were deployed in each pond for 24 h, approximately 30 cm below the water surface and approximately 2 m from the pond edge. At the same period of the passive eDNA sampling, 13 pools, whereby each pool comprised a single pleopod from three individual shrimp, were sampled from each pond. This pooling approach sampled a total of 39 individual shrimp per pond. The shrimp sample size from each pond was selected to provide 95 % confidence of detecting a target prevalence of 10 %, assuming test sensitivity of 0.8 from the pooled tissue samples (epitools.ausvet.com.au).

Table 2
Sampling design utilised for the eDNA sampler submersion duration and farm-scale monitoring studies.

Purpose	Ponds	Shrimp sampled/pond	Tissues sampled/shrimp	eDNA samples/pond	eDNA submersion duration (h)
Submersion duration	$n = 5$	10 (individual)	Pleopod, Hepatopancreas, Gill	10	1, 2, 4, 6 and 24 (duplicate/ pond)
Farm-scale monitoring	$n = 22$	39 (13 pools of 3)	Pleopod	2	24 (duplicate/ pond)

2.4. Passive eDNA sampling

Passive eDNA samplers comprised a hydrophilic, biologically inert, mixed cellulose ester (MCE) microdisc membrane filter composed of cellulose acetate and cellulose nitrate (0.22 μm, 47 mm dia). MCE membranes were selected for the present study on the basis of previously demonstrated eDNA collection performance, particularly in marine environments and for viral adsorption, in addition to their ease of use and availability (Bessey et al., 2021, 2022; Chen et al., 2022; Mejías-Molina et al., 2024a; Schang et al., 2021; Tsuji et al., 2019). To prevent damage or loss of the membrane filters from debris, biota and water current during field deployment, each filter was housed within a plastic housing of 50 mL capacity (Fig. 1). Each housing included six ~1 cm^2 holes to allow water flow across the filter and a ceramic weight to ensure the unit remained submerged during deployment. This approach, similar to those described for successful field deployment of passive eDNA samplers into freshwater creek systems (Kirtane et al., 2020) and human wastewater systems (Schang et al., 2021), facilitated ease-of-use, sterilisation for re-use, and ensured maximum environmental exposure across the membrane.

Duplicate passive samplers were retrieved from each pond after 1, 2, 4, 6 and 24 h of submersion in ponds (termed $eDNA_1$, $eDNA_2$, $eDNA_4$, $eDNA_6$, $eDNA_{24}$, respectively) within the submersion duration study, or 24 h ($eDNA_{24}$) within the farm-scale monitoring study. Upon removal from the pond, membrane filters were collected from the plastic housings and transferred individually using sterile tweezers into DNase and RNase-free tubes containing 2 mL of nuclease-free water (not DEPC-treated) and 2 mL of MagMAX™ CORE Lysis Solution (Applied Biosystems™). Filters were stored (~7 d) at room temperature in lysis solution during sample transport to the laboratory, and within the laboratory, until nucleic acid extraction could be conducted.

Sterile techniques were used for handling of eDNA samples throughout field collection, laboratory processing and analysis. Contamination controls included wearing of gloves to handle all materials and use of sterile, single-use equipment where possible. Membrane filters were certified sterile, and filter housings were sterilised with 10 % bleach solution for 24 h prior to drying and passive sampler assembly. Assembled passive samplers were stored in zip-lock bags until their

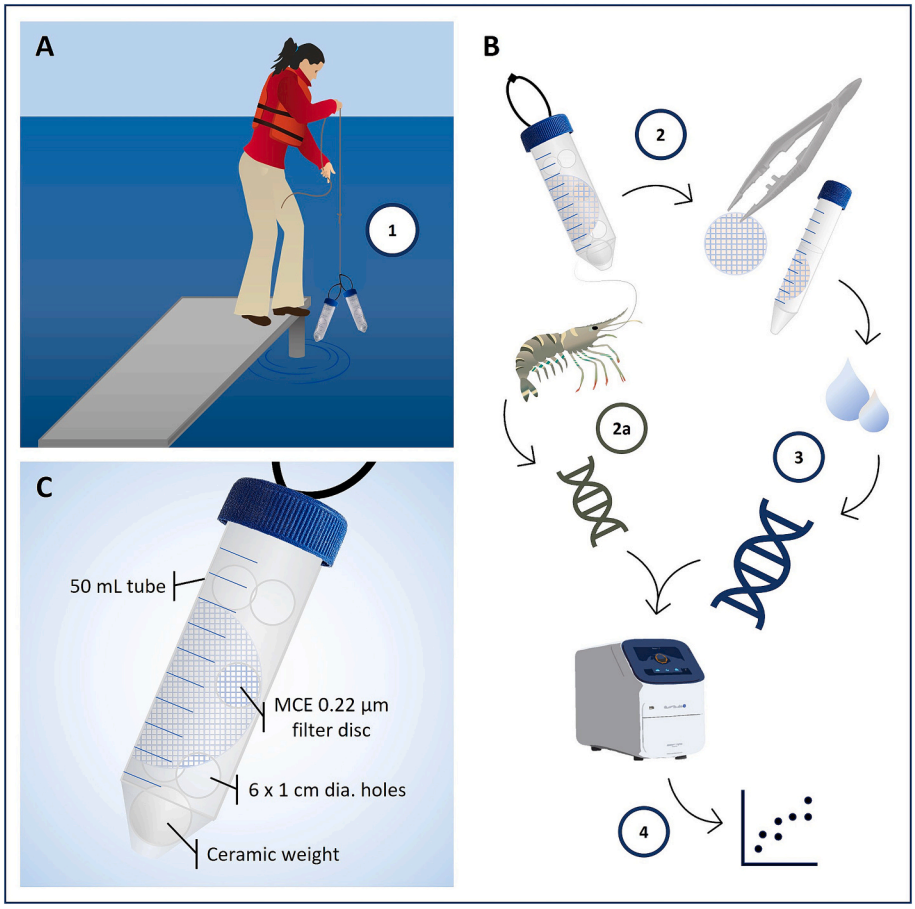

Fig. 1. Illustration of the sampling methodology used in the present study for (A; 1) deploying the passive eDNA sampling devices within commercial *Penaeus monodon* production ponds, (B) retrieving the passive samplers after submersion within the ponds and (2) transferring the filter paper directly into lysis solution. (3) An aliquot of lysis solution was taken for nucleic acid extraction and (4) subsequent qPCR analysis for the detection of IHHNV. From the same shrimp production ponds (2a) shrimp were sampled for complementary analysis for the detection of IHHNV, enabling comparison of eDNA to host detection levels. (C) Diagram of passive sampling device utilised within the study, including a mixed cellulose ester (MCE) membrane filter disc. This figure was created using symbols and images sourced from the Integration and Application Network (www.ian.umces.edu/media-library).

deployment into individual ponds to minimise potential contamination.

2.5. Paired shrimp tissue sampling

A total of 10 shrimp per pond (submersion duration study), or 39 shrimp per pond (farm-scale monitoring), were collected during the same period as the passive eDNA sample collection. For the submersion duration study, shrimp were collected from each pond and stored at -20 °C until further sampling. After thawing at 4 °C, pleopod, gill and hepatopancreas samples were collected from each individual shrimp (n = 50 shrimp). Individual tissue samples were weighed and placed immediately into 2 mL Lysing Matrix D tubes containing 350 μL of MagMAX™ CORE Lysis Solution. For the farm-scale monitoring study, a single 5 mm pleopod clip was sampled from each shrimp (n = 858 shrimp) and placed immediately into 2 mL Lysing Matrix D tubes (MP Biomedicals™) containing 350 μL of MagMAX™ CORE Lysis Solution (Applied Biosystems™) in pools of three pleopods (n = 286 pooled samples). All tissue samples were stored at room temperature in lysis buffer for up to 5 d until nucleic acid extraction could be conducted.

2.6. Nucleic acid extraction

Nucleic acid extraction was conducted as per Arbon et al. (2022) with minor adaptation. Briefly, from tissue samples, 5 μL of 20 mg mL^{-1} MagMAX™ CORE Proteinase K (Applied Biosystems™) was added to each 2 mL Lysing Matrix D tube (MP Biomedicals™) containing the sample in 350 μL of MagMAX™ CORE Lysis Solution (Applied Biosystems™). The sample was homogenised for 30 s at 5000 rpm using a Precellys® 24 tissue homogeniser (Bertin Technologies). From eDNA samples, 5 μL of 20 mg mL^{-1} MagMAX™ CORE Proteinase K was added to each 1.7 mL microcentrifuge tube containing a 350 μL aliquot of lysis solution after incubation with the eDNA filters. Tubes were mixed thoroughly by gentle inversion. The homogenised tissue samples and eDNA samples were incubated at 72 °C for 60 min. Total nucleic acid (TNA) extraction from the homogenised and digested samples was conducted using the MagMAX™ CORE Nucleic Acid Purification Kit (Applied Biosystems™) with a KingFisher™ Flex 96 Deep-Well Magnetic Particle Processor (ThermoFisher Scientific™) as per the manufacturer's instructions. Total nucleic acid was eluted into 100 μL of MagMAX™ CORE Elution Buffer (Applied Biosystems™) and stored at -20 °C until required.

2.7. Quantitative real-time PCR (qPCR)

Quantitative polymerase chain reaction (qPCR) analysis was used for the detection of IHHNV from both the eDNA and tissue samples. Additionally, the *P. monodon* Dicer-1 gene (Dicer-1) was targeted for detection using reverse transcription qPCR (RT-qPCR) as a protocol integrity control for the tissue samples (Arbon et al., 2022). For the detection of IHHNV, the SensiFAST™ Probe Lo-ROX Kit (Meridian Bioscience™) was used. For the detection of Dicer-1, the reverse transcriptase SensiFAST™ Probe Lo-ROX One-Step Kit (Meridian Bioscience™) was used. A total of 1.25 μL of sample template was added to 8.75 μL of the SensiFAST™ Kit master-mix to yield a 10 μL reaction; inclusive of 0.5 μL of the respective target primers (0.2 μM final concentration) and 0.5 μL of target probe (0.05 μM final concentration). IHHNV and Dicer-1 primer and probe details are listed in Table 3.

For the submersion duration study, three 1.25 μL aliquots of each TNA extract were tested as technical replicates for each target. For all samples, analysis was conducted using a QuantStudio™ 5 Real-Time PCR System, 384-well (Applied Biosystems™). Cycle conditions were standardised for all assays, consisting of a reverse-transcription step of 45 °C for 10 min, initial denaturation at 95 °C for 5 min, followed by 45 cycles of denaturation at 95 °C for 10 s and annealing and extension at 60 °C for 30 s. Raw data were processed using QuantStudio™ Design & Analysis Software v1.5.2 (ThermoFisher Scientific™), using the ΔRn method with a relative threshold of 0.05, to produce cycle threshold (Ct) values of positive detections. Samples which did not amplify beyond the relative threshold within 45 cycles were categorised as undetermined. Positive IHHNV and Dicer-1 template controls and a no template control were included in each analysis.

IHHNV detection data was standardised to calculated copy number μL^{-1} TNA extracted. To do so, a serial 10-fold dilution of a gBlock™ Gene Fragment (Integrated DNA Technologies™) was used as a synthetic linear dsDNA template for IHHNV (Supplementary file S5). Copy number (μL^{-1}) was calculated for each of the gBlock™ dilutions in the series. The gBlock™ Gene Fragment was designed to comprise the IHHNV genomic sequence targeted by the qPCR assay described in Table 3, including flanking sequence derived from sequences available on the National Centre for Biotechnology Information (NCBI) database. Each point on the 10-fold dilution series was analysed in triplicate by qPCR. The dilution series was used to generate a standard curve ($R^2 > 0.99$), from which the conversion of Ct value to target copy number (μL^{-1}) could be estimated.

From the qPCR analysis of shrimp tissue and eDNA, samples categorised as undetected were assumed to have a target copy number of zero. For the submersion duration study, target copy number μL^{-1} TNA extract was converted to target copy number mg^{-1} tissue for pleopod, gill and hepatopancreas samples to enable relative comparison between the tissue samples.

2.8. qPCR inhibition testing

Passively collected eDNA samples from all ponds across both the submersion duration study and the farm-scale monitoring were additionally tested for inhibition using a DNA exogenous quantitative internal positive control (IPC). Inhibition of qPCR reactions were assessed using Photorhabdus insect-related toxin subunit A (Pir-A) gene template as a DNA IPC. The eDNA samples were first confirmed negative for Pir-A using the assay described by Han et al., 2015 listed in Table 3, and the

Table 3
Quantitative polymerase chain reaction (qPCR) primer and probe details as used in the present study.

Target	Primer/ Probe	Sequence (5'-3')	Length [bp]	Product length [bp]	Reference
Infectious hypodermal and haematopoietic necrosis virus (IHHNV)	IHHNV 309 qF	CCTAAAGAAAACAGTGCAGAATAT	24	98	(Cowley et al., 2018)
	IHHNV 309 qR	TCATCGTCAAGTTTATTGACAAGTTC	26		
	IHHNV 309 qProbe	CTCCAACACTTAGTCAAA	18		
Penaeus monodon Dicer-1 gene (Dicer-1)	Dicer-1 qF	TGGTACCAAAGTCACCCATTAG	22	91	(Su et al., 2008)
	Dicer-1 qR	ACCTTCCCATCAACAAGACGTT	22		
	Dicer-1 qProbe	AACCAGAAACAGCCAAAT	18		
Photorhabdus insect-related toxin subunit A (Pir-A) gene (exogenous, quantitative internal positive control target)	Pir-A qF	TTGGACTGTCGAACCAAACG	20	135	(Han et al., 2015)
	Pir-A qR	GCACCCCATTGGTATTGAATG	21		
	Pir-A qProbe	AGACAGCAAACATACACCTATCATCCCGGA	30		

same qPCR analysis conditions as described for the detection of IHHNV. For the inhibition test, 1.25 μL of IPC was spiked into each eDNA sample well, inclusive of 1.25 μL of eDNA sample template and 8.75 μL of master-mix. A negative control without both IPC and eDNA sample template ($n = 3$), and a negative control with IPC and 1.25 μL of DNase- and RNase-free water (no-eDNA template control) ($n = 14$) were included on the qPCR reaction plate. Inhibition of eDNA samples was assessed by comparing amplification of IPCs within spiked eDNA samples to that of IPCs within the no-eDNA template controls to evaluate the quantitative shift in cycle threshold (Ct) value. A $\Delta Ct \geq 3$ cycles beyond the average Ct of the no-eDNA template controls were considered substantially inhibited (Hartman et al., 2005).

2.9. Statistical analysis

Statistical analysis and data visualization was conducted using R (R Core Team, 2022). Descriptive statistical parameters, including total number of samples tested, total number of positive detections, rate of positive detection (%) and the average (mean) and standard deviation (SD) of positive detections in copy number μL^{-1}, or copy number mg^{-1} were calculated for averaged technical replicates of each target, between sample types and between ponds. Further statistical analysis to evaluate the differences in copy number and correlation of detection between sample types were conducted using $\log_{10}$ transformed copy number μL^{-1} or mg^{-1}, after zero values were adjusted to one. Significant differences in overall IHHNV detection between the sample types were determined using a Kruskal-Wallis test (R Core Team, 2022), due to significant deviation from homogeneous variances, as determined via a Levene's test (R package: *car*; $\alpha = 0.05$; Fox and Weisberg, 2019). Post-hoc analysis was run using a Dunn test with Holm's correction (R package: *FSA*; $\alpha = 0.05$; Ogle et al., 2022).

To evaluate how well quantitative IHHNV detection from passively sampled eDNA reflected the level of IHHNV detected in shrimp tissue samples, the ratio of detected IHHNV concentration between eDNA and shrimp tissue were calculated per pond, for each submersion duration. Similar to the accumulation ratios described by Habtewold et al., 2022, the detection ratios were calculated as $C_{eDNA(t)}/C_{shrimp}$, where $C_{eDNA(t)}$ = mean $\log_{10}$ IHHNV copies μL^{-1} detected in eDNA samples at submersion time t, and C_{shrimp} = mean $\log_{10}$ IHHNV copies μL^{-1} (or mg^{-1}; submersion duration study) detected in complementary shrimp tissue samples collected from the same pond.

For the submersion duration study, linear regression analysis was used to estimate the relationship of quantitative IHHNV detection from shrimp tissue and passive eDNA samples collected from each pond. IHHNV detection patterns from Pond A differed notably from those of Ponds B-E. Therefore, linear regression analyses were performed on tissue and eDNA IHHNV detection data both including all ponds, and excluding Pond A, for comparison. The correlative significance of linear relationships between parameters were determined using Pearson's correlation analysis (R package: *Hmisc*; $\alpha = 0.05$; Harrell, 2023).

For the farm-scale study, the relationship between mean $\log_{10}$ IHHNV copies μL^{-1} detected from pooled pleopod tissue samples and passive $eDNA_{24}$ samples from individual ponds was assessed using multiple regression models. Specifically, linear, second-degree polynomial and logarithmic models were fitted to the data using ordinary least squares (R Core Team, 2022). Additionally, a Michaelis-Menten model (Eq. 1), often used to describe non-linear eDNA saturation and decay kinetics (Coulter et al., 2019; Curtis and Larson, 2020), was fitted to the data using nonlinear least squares (nls) regression (R package: *minpack.lm*; $\alpha = 0.05$; Elzhov et al., 2023).

Equation 1. Michaelis-Menten model equation describing the relationship of IHHNV concentration detected from $eDNA_{24}$ samples, and shrimp tissue samples collected from the same ponds.

$$eDNA = \frac{V_{max} \times S}{K_m + S} \quad (1)$$

Where *eDNA* is the mean $\log_{10}$-transformed detection of IHHNV copies μL^{-1} from duplicate $eDNA_{24}$ samples, S is the mean $\log_{10}$-transformed detection of IHHNV copies μL^{-1} from pooled shrimp pleopod samples ($n = 13$ pools per pond), V_{max} is the theoretical maximum rate of eDNA IHHNV detection within the pond environment, assuming no further increase in response regardless of addition tissue detection (saturation point), and K_m is the half-saturation constant, representing the tissue detection concentration where eDNA detection reaches half of V_{max}, providing insight into how quickly the response approaches the asymptote.

Within the observed detection range, the Michaelis-Menten model was well-approximated by a second-degree polynomial constrained with a zero-intercept. To facilitate calculation and comparison of statistical parameters across models, the polynomial approximation of the Michaelis-Menten model was used as a proxy for the Michaelis-Menten model. Model selection was informed by the adjusted R squared (R^2_{adj}), Bayesian information criterion (BIC) and Akaike information criterion (AIC), alongside visual assessment of each model's goodness-of-fit.

Generalised linear modelling (R Core Team, 2022) was used to assess the relationship between various pond water quality parameters and IHHNV mean copy number detected from $eDNA_{24}$ samples across the farm monitoring study. The model incorporated predictors including mean $\log_{10}$ transformed IHHNV copies μL^{-1} in pooled pleopod tissue, days of culture on day of sampling (DOC) and pond pH (AM and PM values), temperature (AM and PM values), dissolved oxygen (DO; AM and PM values), and salinity.

3. Results

3.1. qPCR inhibition

Substantial inhibition of eDNA samples was qualified by a $\Delta Ct \geq 3$ between the detected Ct of the IPC in spiked eDNA samples and the mean detected IPC Ct within the no-eDNA template controls (IPC Ct mean ± SD = 25.56 ± 0.16) (Hartman et al., 2005; Sieber et al., 2020). Substantial inhibition of the IPC ($\Delta Ct \geq 3$) was not observed for any of the eDNA samples collected (IPC $|\Delta Ct|$ mean ± SD = 0.25 ± 0.36).

The IHHNV positive template control was consistently detected across all qPCR test plates, with less than a one cycle threshold (Ct) value variation across all detections (maximum $|\Delta Ct| = 0.98$, mean ± SD Ct = 26.15 ± 0.27). No amplification was detected from the no template controls.

3.2. Submersion duration study

IHHNV was detected in all shrimp tissue and eDNA samples, from all five ponds analysed for the submersion duration study (Table 4). The IHHNV load in shrimp tissues was variable across the five ponds, with Pond B yielding the highest mean IHHNV loads, followed by Pond D > Pond E > Pond C > Pond A. Across all ponds, IHHNV detection between tissue types of individual shrimp were not significantly different ($p < 0.05$). However, overall detected IHHNV copies mg^{-1} were highest from pleopod, followed by gill, and hepatopancreas. Detections between pleopod and gill tissue ($R^2 = 0.94$), pleopod and hepatopancreas tissue ($R^2 = 0.92$), and gill and hepatopancreas tissue ($R^2 = 0.93$) of individual shrimp were significantly linearly correlated ($p < 0.001$).

Across all submersion durations (1, 2, 4, 6 and 24 h), there were no significant differences in eDNA IHHNV detection between ponds ($p > 0.05$). However, across all ponds, quantitative detection of IHHNV from the passive eDNA samples increased with increasing submersion duration, with submersion for 24 h yielding the highest overall detected copy number μL^{-1} and significantly higher detection than eDNA samples submerged for 1 h ($p = 0.046$).

To evaluate how well quantitative IHHNV detection from passively sampled eDNA reflected IHHNV detection in shrimp tissue samples, the mean $\log_{10}$ IHHNV copies μL^{-1} detected in eDNA samples at each

Table 4
Individual pond data for IHHNV qPCR detection within the passive sampler submersion duration study. The data is presented as pond-level summaries, including IHHNV detection from duplicate passive eDNA samples submerged for 1, 2, 4, 6 and 24 h ($eDNA_{1, 2, 4, 6, 24}$), and individual shrimp pleopod, gill and hepatopancreas samples ($n = 10$ shrimp per pond) collected from *Penaeus monodon* production ponds ($n = 5$). IHHNV calculated copies are presented per µL of TNA extract for eDNA samples, and per mg of tissue for pleopod, gill and hepatopancreas samples. Superscript letters indicate significant differences between ponds for shrimp tissues. Statistical comparison between submersion durations for individual ponds was not possible due to the small sample sizes, and there were no significant differences between ponds for submersion durations for eDNA samples. All sample replicates across all ponds were positive for the detection of IHHNV. Samples were collected during the 2022/2023 production season on a commercial shrimp farm in North Queensland, Australia.

		Pond ID				
	Sample type	A	B	C	D	E
IHHNV copies mg^{-1} (mean ± SD)	Pleopod	$1.54 \times 10^3 \pm 1.39 \times 10^3$	$7.96 \times 10^7 \pm 9.32 \times 10^7$	$6.05 \times 10^6 \pm 1.89 \times 10^7$	$6.05 \times 10^7 \pm 6.04 \times 10^7$	$1.20 \times 10^7 \pm 2.61 \times 10^7$
	Gill	$5.07 \times 10^2 \pm 3.89 \times 10^2$	$5.00 \times 10^7 \pm 4.15 \times 10^7$	$5.89 \times 10^5 \pm 1.75 \times 10^6$	$2.31 \times 10^7 \pm 3.63 \times 10^7$	$1.75 \times 10^7 \pm 4.72 \times 10^7$
	Hepatopancreas	$5.93 \times 10^1 \pm 1.1 \times 10^2$	$2.10 \times 10^7 \pm 1.81 \times 10^7$	$2.48 \times 10^5 \pm 7.34 \times 10^5$	$2.12 \times 10^7 \pm 5.44 \times 10^7$	$1.84 \times 10^6 \pm 4.05 \times 10^6$
IHHNV copies μL^{-1} (mean ± SD)	$eDNA_1$	2.61 ± 0.68	$1.95 \times 10^2 \pm 3.95 \times 10^1$	$2.05 \times 10^1 \pm 7.31$	$1.00 \times 10^2 \pm 1.02 \times 10^1$	$6.03 \times 10^1 \pm 1.15 \times 10^1$
	$eDNA_2$	2.14 ± 0.18	$3.15 \times 10^2 \pm 6.53 \times 10^1$	$2.84 \times 10^1 \pm 5.49$	$2.22 \times 10^2 \pm 3.91 \times 10^1$	$1.16 \times 10^2 \pm 2.29 \times 10^1$
	$eDNA_4$	6.77 ± 3.13	$3.94 \times 10^2 \pm 1.20 \times 10^2$	$5.45 \times 10^1 \pm 2.19 \times 10^1$	$3.25 \times 10^2 \pm 7.52 \times 10^1$	$1.58 \times 10^2 \pm 2.64$
	$eDNA_6$	$3.24 \times 10^1 \pm 2.81 \times 10^1$	$9.95 \times 10^2 \pm 4.11 \times 10^2$	$9.95 \times 10^1 \pm 2.78 \times 10^1$	$3.12 \times 10^2 \pm 7.88$	$2.26 \times 10^2 \pm 1.77 \times 10^1$
	$eDNA_{24}$	$3.18 \times 10^2 \pm 3.00 \times 10^1$	$1.41 \times 10^3 \pm 3.93 \times 10^2$	$2.05 \times 10^2 \pm 2.76 \times 10^1$	$1.07 \times 10^3 \pm 1.16 \times 10^2$	$5.03 \times 10^2 \pm 1.30 \times 10^2$
Log_{10}(IHHNV copies mg^{-1}) (mean ± SD)	Pleopod	3.01 ± 0.44^{a}	7.46 ± 0.8^{b}	4.92 ± 1.08^{ac}	7.45 ± 0.73^{b}	6.13 ± 0.87^{bc}
	Gill	2.6 ± 0.33^{a}	7.39 ± 0.7^{b}	4.6 ± 0.86^{ac}	6.72 ± 0.99^{b}	5.74 ± 1.11^{bc}
	Hepatopancreas	1.36 ± 0.58^{a}	6.96 ± 0.76^{b}	4.01 ± 1^{ac}	6.28 ± 1.08^{b}	5.1 ± 1.03^{bc}
Log_{10}(IHHNV copies μL^{-1}) (mean ± SD)	$eDNA_1$	0.41 ± 0.12	2.28 ± 0.09	1.3 ± 0.16	2 ± 0.04	1.78 ± 0.08
	$eDNA_2$	0.33 ± 0.04	2.49 ± 0.09	1.45 ± 0.08	2.34 ± 0.08	2.06 ± 0.09
	$eDNA_4$	0.81 ± 0.21	2.58 ± 0.13	1.72 ± 0.18	2.51 ± 0.1	2.2 ± 0.01
	$eDNA_6$	1.41 ± 0.44	2.98 ± 0.18	1.99 ± 0.12	2.49 ± 0.01	2.35 ± 0.03
	$eDNA_{24}$	2.5 ± 0.04	3.14 ± 0.12	2.31 ± 0.06	3.03 ± 0.05	2.69 ± 0.11

submersion time were considered as a proportion of the mean log_{10} IHHNV copies mg^{-1} detected in complementary shrimp pleopod samples collected from the same pond (IHHNV detection ratio (eNA/pleopod); Fig. 2). Ponds B-E exhibited a consistent linear increase in the IHHNV detection ratio with increasing submersion duration, demonstrating quantitative detection from eDNA that steadily approached the loading detected from shrimp pleopod tissues (i.e., a ratio of 1). After 1 h of submersion, IHHNV loadings detected in eDNA were 28 % ± 2 % of the mean loading mg^{-1} detected in shrimp pleopod tissues sampled from Ponds B, C, D and E (Fig. 2). The ratio for Ponds B-E increased to 43 % ± 3 % after 24 h of submersion. In contrast, Pond A displayed a notably different pattern in the IHHNV detection ratio over the range of submersion durations. Detection of IHHNV from Pond A at $eDNA_1$ and $eDNA_2$ were lower than expected, representing 14 % and 10 % of the mean loading detected from shrimp pleopod tissue, respectively. However, from 4 h ($eDNA_4$) onward, the proportion of IHHNV detected in eDNA rapidly increased to 83 % of that detected in shrimp pleopods after 24 h. Due to this anomalous detection pattern, when analysing the pond-wise linear correlation of IHHNV detection between eDNA and shrimp tissues at the various submersion durations, analysis was conducted both with, and without, Pond A (Fig. 2).

Strong linear correlations were observed between mean log_{10} IHHNV copies mg^{-1} detected in shrimp pleopod tissue and the mean log_{10} IHHNV copies μL^{-1} detected in eDNA samples submerged for 1, 2, 4, 6 and 24 h ($eDNA_{1\text{-}24}$) across all ponds (R^2_{adj}: 0.57–0.98). Correlation coefficients and statistical significance were reduced for $eDNA_{1\ -\ 6}$ when detections from Pond A were excluded, however, $eDNA_{24}$ increased considerably from $R^2_{adj} = 0.57$ to 0.98. The high correlation observed between pleopod and eDNA IHHNV detection was also observed for gill and hepatopancreas tissues, given the high correlation of IHHNV detection between pleopod, gill and hepatopancreas tissues of individual shrimp (Fig. S1).

3.3. Farm-scale monitoring

Based on the detection results of the submersion duration study, the farm-scale monitoring study deployed passive samplers for 24 h of submersion across all 22 ponds. IHHNV was detected in all ponds by shrimp tissue analysis (Table 5). Detection rates ranged between 31 % (4/13) and 100 % (13/13) for pools of 3 shrimp pleopods, sampled from individual ponds (Table S4). IHHNV was detected in 95 % (21/22) of the ponds by one (9 %; 2/22) or both (86 %; 19/22) duplicate $eDNA_{24}$ samples. Agreement between tissue and $eDNA_{24}$ samples in identifying ponds as IHHNV positive by qPCR was observed for 95 % (21/22) of the ponds. In a single pond (1/22), IHHNV was detected in shrimp tissue, but not in $eDNA_{24}$ samples. Within the three ponds which yielded either one or neither of the duplicate $eDNA_{24}$ samples positive for IHHNV, all had average IHHNV loading of less than 10 copies μL^{-1} in pooled shrimp pleopod samples (Fig. 3).

Overall, IHHNV detection from pooled pleopod tissue averaged $3.44 \times 10^6 \pm 8.71 \times 10^6$ copies μL^{-1}, and $1.74 \times 10^4 \pm 4.86 \times 10^4$ copies μL^{-1} from $eDNA_{24}$ samples (Table 5). However, mean log_{10} transformed IHHNV copies μL^{-1} were not significantly different between pooled pleopod tissue samples and $eDNA_{24}$ samples ($H_{df=1} = 2.9$, $p = 0.08$).

From ponds where all pooled pleopod tissue samples ($n = 13$ samples $pond^{-1}$) were positive for IHHNV ($n = 17$ ponds), a negative linear correlation ($R^2 = 0.41$, $p = 0.004$) was observed between the detection ratio and mean IHHNV detection in pooled shrimp pleopod tissue (Fig. S2), indicating that eDNA detection was increased in ponds with higher pleopod tissue detection, but at a diminishing rate relative to the increase in pleopod detection. Accordingly, the relationship between mean log_{10} IHHNV copies μL^{-1} detected from pooled pleopod tissue samples and passive $eDNA_{24}$ samples from individual ponds was best described by a Michaelis-Menten model, commonly used to describe eDNA saturation or decay kinetics (Coulter et al., 2019; Curtis and Larson, 2020). Nonlinear least squares regression determined the parameters V_{max} (theoretical maximum rate of eDNA IHHNV detection

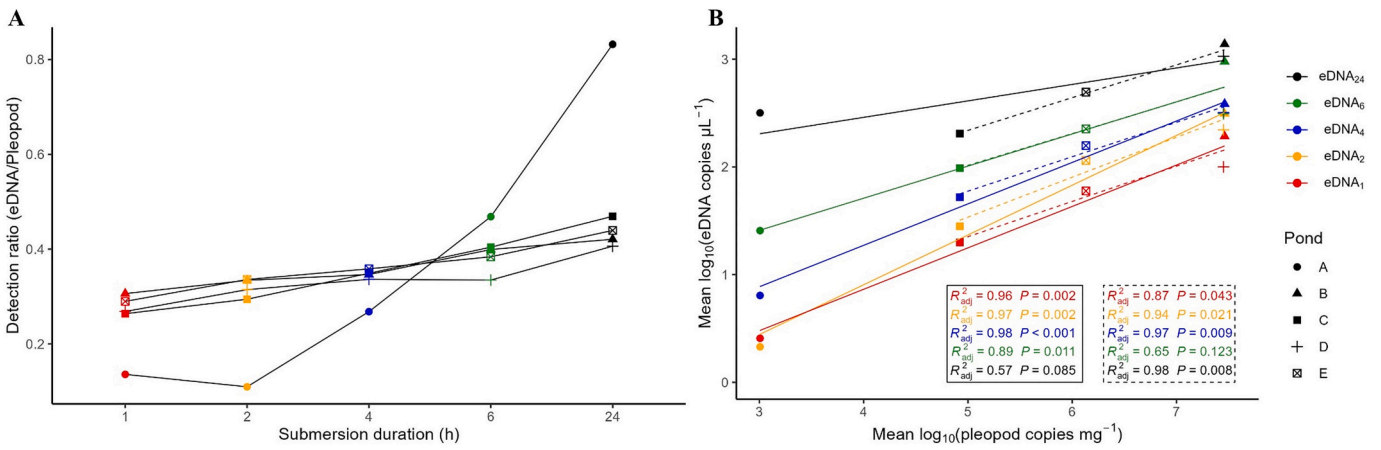

Fig. 2. (A) IHHNV detection ratio between passively sampled eDNA submerged in shrimp ponds for 1, 2, 4, 6 and 24 h and *Penaeus monodon* pleopods sampled from the same ponds, where the ratio is defined as the mean $\log_{10}$ IHHNV copies μL^{-1} detected in duplicate eDNA samples at each submersion time divided by the mean $\log_{10}$ IHHNV copies mg^{-1} detected in corresponding shrimp pleopod samples ($n = 10$ shrimp per pond). Detection ratios calculated for each pond (A-E) over time are connected by lines. (B) Correlation of IHHNV detection between duplicate passive eDNA samples after 1, 2, 4, 6 or 24 h of submersion ($eDNA_{1, 2, 4, 6, 24}$) and *P. monodon* pleopod samples ($n = 10$ per pond) collected from the same production ponds (Ponds A-E). Adjusted Pearson's correlation coefficient (R^2_{adj}; Holm corrected) and *p*-values were derived from linear correlation calculated between eDNA and shrimp tissue detection across all ponds ($n = 5$, A-E) for each eDNA submersion duration (solid line). Linear correlation was also calculated for Ponds B-E (i.e. excluding Pond A) (dashed line). (A) and (B) share common legends. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 5
Summary of IHHNV qPCR detection within the farm-scale monitoring study. The number of ponds, and total number of samples positive for IHHNV by duplicate passive eDNA samples submerged for 24 h, and complementary pooled *Penaeus monodon* pleopod tissue samples ($n = 13$ pools of three individual shrimp pleopods per pond) collected from the same commercial production ponds ($n = 22$) are detailed. Mean ± standard deviation (SD) of original and $\log_{10}$ transformed IHHNV calculated copy number are presented μL^{-1} of TNA extract for each sample type.

Sample Type	Ponds positive	Total samples positive	Copies μL^{-1} (mean ± SD)	Log_{10}(copies μL^{-1}) (mean ± SD)
Pooled pleopod	100 % (22/22)	86 % (256/286)	$3.44 \times 10^6 \pm 8.71 \times 10^6$	3.62 ± 2.55
$eDNA_{24}$	95 % (21/22)	91 % (40/44)	$1.74 \times 10^4 \pm 4.86 \times 10^4$	2.41 ± 1.70

within the pond environment; 'saturation point') = 13.1, and K_m (the half-saturation constant, representing the tissue detection concentration where eDNA detection reaches half of V_{max}) = 14.2. The relationship between these two parameters ($K_m > V_{max}$), suggests that within the observed range of detections, saturation effects did not have a large impact on eDNA detections. To enable the calculation and interpretation of statistical parameters for this relationship, the model was approximated by a second-degree polynomial with an intercept of zero (Fig. 3). From the polynomial approximation, a strong correlation of mean IHHNV detection in ponds between eDNA and shrimp tissue was observed ($R^2 = 0.95$, $F_{2,20} = 220$, $p < 0.001$; Fig. 3).

3.4. Pond water quality

Pond water quality was recorded across all ponds assessed within this study (Table S3). Generalised linear modelling was used to assess the relationship between various pond water quality parameters and IHHNV mean copies μL^{-1} detected from $eDNA_{24}$ samples across the farm monitoring study. The predictor variables included mean IHHNV copies μL^{-1} from pooled pleopod tissue from each pond, days of culture (DOC) and pond pH (AM and PM values), temperature (AM and PM values), dissolved oxygen (DO, AM and PM values), and salinity. The mean IHHNV copies μL^{-1} from pooled pleopod tissue was the most significant predictor of IHHNV copies μL^{-1} detected from pond $eDNA_{24}$ samples ($p < 0.001$), confirming the strong positive correlation between pond shrimp tissue and $eDNA_{24}$ IHHNV detection. The AM pH value also yielded a significant positive association with $eDNA_{24}$ IHHNV detection ($p = 0.025$), indicating that higher morning pH values were associated with higher detection of IHHNV from eDNA samples. Other variables, including PM pH, temperature, DO, DOC, and salinity, were not statistically associated with detection of IHHNV in $eDNA_{24}$ samples ($p > 0.05$).

4. Discussion

The present study investigated the utility of passive eDNA sampling for the detection and quantification of viral shrimp pathogens from commercial *P. monodon* aquaculture production ponds. To our knowledge, this is the first study applying passive sampling for eDNA detection of viruses in shrimp aquaculture. The findings demonstrate that IHHNV can be detected from passive eDNA samples. The detection results of IHHNV indicate that adequate calibration studies may enable proxy quantification of shrimp host infection using eDNA. This study also demonstrated that IHHNV could be detected from eDNA samples submerged in shrimp production ponds for only 1 h, and that detected copy number increased with increasing submersion duration. These results highlight two potential application strategies for eDNA sampling from shrimp ponds, including rapid sampling for estimation of pond level pathogen presence and quantification, and extended sampling for pathogen surveillance.

4.1. qPCR inhibition testing

The presence of inhibitors within environmental samples are considered a significant challenge for the application of eNA-based detection (Goldberg et al., 2016; Rieder et al., 2024). Addition of a quantitative exogenous IPC within analysis of eNA samples enables the assessment and potential calibration for inhibitor effects to improve the representativeness of eNA signal to host infection and determine appropriate data-associated caveats (Bowers et al., 2021). Across all eDNA samples analysed in this study, inhibition of qPCR analysis was not detected, as demonstrated by consistent detection of the exogenous quantitative IPC. Complementary sampling and analysis of host tissue was also performed to verify the eDNA detection results (Sieber et al.,

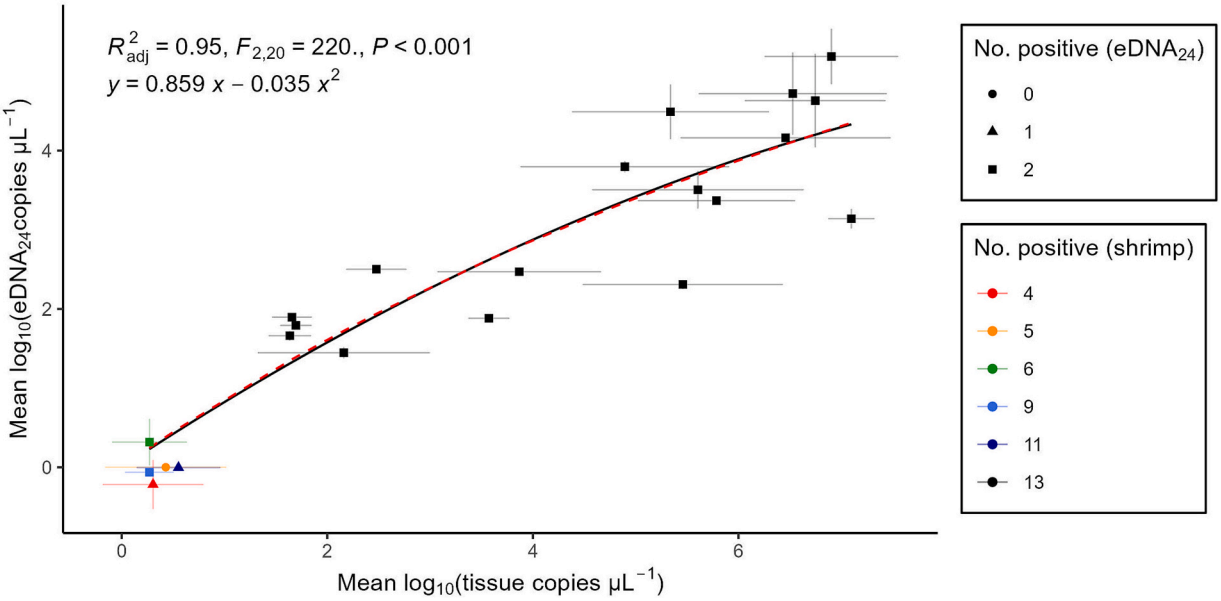

Fig. 3. Comparative qPCR detection of IHHNV from passive $eDNA_{24}$ samples and pooled *Penaeus monodon* pleopod tissue samples collected from commercial production ponds (n = 22). Each point represents the mean log_{10} transformed copy number μL^{-1} detected from 13 pools of three individual shrimp pleopods compared to the mean log_{10} transformed copy number μL^{-1} detected from duplicate $eDNA_{24}$ samples collected from the same pond. Standard deviation of IHHNV detection for each sample type from each pond are displayed as horizontal (pooled pleopod) or vertical ($eDNA_{24}$) lines radiating from each point. Pearson's correlation coefficient (R^2_{adj}) and test statistics are displayed for the polynomial approximation (black line) of the Michaelis-Mentel model (red dashed line) describing the line of best fit. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2024), further supporting the reliability of the eDNA-based detections.

4.2. Submersion duration study

IHHNV copies μL^{-1} detected from eDNA samples increased with longer submersion times, reaching significantly higher detection after 24 h compared to 1 h. The increased detection with 24 h submersion indicates that target eDNA adsorption continued to occur over time with prolonged exposure of the sampling unit. Comparisons with previous studies deploying cellulose ester membrane filters for passive eDNA collection highlight that eDNA detection responses to submersion duration vary by environment and detection target. For example, in a 3 million L marine aquarium containing 50 known fish species, significant increases in fish eDNA detection were not observed with longer submersion times of passive eDNA samples from 5 min to 18 h (Bessey et al., 2022). Similarly, no significant increase in eDNA detection was observed for the Chinese giant salamander (*Andrias davidianus*) from passive samples submerged for 30 min to 72 h in 15 L freshwater tanks, each housing a single animal (68-72 g) (Chen et al., 2022). In contrast, fish eDNA detection from passive eDNA samples deployed in an open tropical reef environment increased between 4 and 8 h of submersion, reaching equilibrium after 8 h and remaining steady until 24 h (Bessey et al., 2021). When applied to viral detection, Mejías-Molina et al. (2024a) observed that nitrocellulose membranes adsorbed increasing amounts of human adenovirus (HAdV) and pepper mild mottle virus (PMMoV) over time (1, 3, 7 and 10 d) from groundwater (50 L) spiked with 1 mL of human wastewater (1:100), with maximum virus adsorption occurring between 7 and 10 d. The variability in detection response to submersion time reported across these studies indicate that environment- and target-specific factors likely influence the dynamics of eDNA capture, saturation, and degradation on filter material during passive sampling, thereby impacting the effectiveness of different filter types and submersion durations for different settings (Kirtane et al., 2020; Mejías-Molina et al., 2024a; Wang et al., 2021).

In the present study, although longer submersion time from 1 to 24 h yielded increasing IHHNV detection, IHHNV copies detected from eDNA (in all submersion times) correlated strongly with IHHNV loads in shrimp tissues. Therefore, shorter submersion durations may be sufficient to indicate shrimp infection levels in ponds with high prevalence and host tissue loads, and may be suitable for rapid, large-scale estimation of host infection levels in ponds where infection is suspected or known. Although further investigation is required, the short 1 h submersion time presents a potential breakthrough tool for rapid risk evaluation of production ponds in response to a disease outbreak.

In our study, extended submersion of passive samplers within shrimp ponds yielded higher detected IHHNV loads, indicating that longer submersion (up to 24 h) may enhance detection sensitivity. Longer submersion of passive eDNA samplers is likely to be more suitable for surveillance purposes, particularly for detecting low-prevalence targets (Chen et al., 2022). While these findings indicate a strong utility for passive eDNA sampling as a surveillance tool for shrimp pathogens, further studies are necessary to elucidate detection dynamics over time in ponds with low infection levels to fully validate the eDNA approach. In the present study, a single pond (Pond A) exhibited a considerably different pattern in IHHNV detection over the range of submersion durations. Notably, shrimp tissue IHHNV loads detected from Pond A were the lowest among the five ponds assessed. It is unclear whether the detection patterns observed from Pond A were characteristic of ponds with low host infection levels, or were influenced by confounding factors, such as an unrecorded modification to the pond environment (e.g. mechanical aeration influencing total suspended solids) (Hayes et al., 2022). To address these uncertainties, further analyses in ponds with comparable or lower IHHNV loading are required.

The eDNA signal captured by passive samplers in this study likely represents a combination of intact viral particles, free viral DNA, and virus-containing cellular material (Bessey et al., 2022; Dominic et al., 2023; Power et al., 2023). While our findings suggest strong correlation with tissue-based detection, the specific composition of viral material on the filters remains undetermined. Further work is needed to characterise the physical state and infectivity of IHHNV material collected via passive eDNA sampling.

4.3. Farm-scale monitoring

The farm-scale eDNA monitoring approach demonstrated that passive eDNA collection for large surveillance-type sampling efforts was both practical and had comparable detection performance to current industry standard tissue sampling. Passively collected eDNA samples incubated for 24 h yielded representative positive detection of IHHNV, with agreement between tissue and $eDNA_{24}$ samples in identifying ponds as IHHNV positive by qPCR in 95 % (21/22) of the ponds analysed. Ponds with average IHHNV shrimp tissue detection greater than 10 copies μL^{-1} and 85 % (11/13) of the pooled samples positive consistently produced positive IHHNV detection from both duplicate passive samplers deployed. Below this threshold, environmental IHHNV eDNA levels may be approaching the limit of detection (Schang et al., 2021). While the lower limits of detection from passive eDNA samples in this context are yet to be elucidated, the deployment of replicate samplers in shrimp production ponds is recommended to improve the likelihood and representativeness of detection at low target presence (Mejías-Molina et al., 2024a).

4.4. Calibration of eDNA signal to host infection

Although eDNA approaches provide substantial advantages for sampling scope and efficiency, the disconnect between the infected host and the environmental sample presents challenges for translating eDNA detection results to evaluate potential host disease risk and to inform biosecurity management decisions (Bass et al., 2023; Bessey et al., 2022). Currently, only one study, involving eDNA detection of a WOAH listed aquatic disease agent (*Gyrodactylus salaris* hosted by Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*)), has demonstrated sufficient validation for inclusion in the Manual of Diagnostic Tests for Aquatic Animals (Rusch et al., 2018; WOAH, 2022; 2021).

For target detection from eDNA samples to be valuable in monitoring host infection quantitatively, calibration studies are necessary to demonstrate statistical associations between detection from eDNA and host derived samples (Bass et al., 2015; WOAH, 2022). To date, few studies that have applied active or passive eDNA collection approaches in aquatic contexts have generated statistically supported associations between eDNA detections and detections in the host organism. Richey et al. (2020) observed a significant association between the presence of the myxozoan-associated enteronecrosis agent *Ceratonova shasta* from actively filtered eDNA samples and its prevalence in fish hosts within freshwater rivers. Building upon the findings of Strand et al. (2014), Wittwer et al. (2019) demonstrated a significant ($R^2 = 0.53$) non-linear correlation between spore concentrations of the crayfish plague agent *Aphanomyces astaci* estimated via active eDNA collection and those detected in infected crayfish tissue sampled from freshwater streams using traditional trapping methods. In the present study, we compared quantitative IHHNV detection in shrimp tissue samples with IHHNV detection from passively collected eDNA incubated for 24 h in shrimp ponds. IHHNV loads detected from eDNA were positively correlated with average IHHNV detections from pooled shrimp pleopod tissues sampled from the same ponds ($R^2 = 0.95$).

The relationship between eDNA and shrimp tissue IHHNV detection in this study was best described by a non-linear Michaelis-Menten model, indicating that eDNA detection increased with higher IHHNV detection in shrimp tissue, but exhibited diminishing rates of increase at higher shrimp detection levels. This trend aligns with the broader application of non-linear saturation and decay models in describing eDNA concentrations derived from qPCR detection and host abundance across aquatic species including ayu sweetfish (*Plecoglossus altivelis*) ($0.80 \leq R^2 \leq 0.98$; Doi et al., 2017), common carp (*Cyprinus carpio*) ($R^2 = 0.87$; Doi et al., 2015), silver carp (*Hypophthalmichthys molitrix*) ($R^2_{McFadden's} = 0.34$; Coulter et al., 2019) and, common spadefoot toad (*Pelobates fuscus*) ($R^2 = 0.68$; Thomsen et al., 2012). Within our study, the slightly reduced efficiency of eDNA recovery relative to the increase in host tissue concentration may have been contributed to by saturation of the membrane filters (Jones et al., 2022) or decay of adsorbed eDNA during deployment, resulting from high water temperatures and microbial activity within the shrimp ponds (Zulkefli et al., 2019). Competitive binding of the eDNA to other organic matter within the pond environment may have also limited the adsorption of eDNA to the filters (Barnes et al., 2021), although these factors were not directly assessed within our study. Notably, the fitted Michaelis-Menten model exhibited a far shallower asymptote (where $K_m > V_{max}$), compared to those observed in other studies, such as Coulter et al. (2019) and Wittwer et al. (2019), suggesting that either saturation or decay effects were less pronounced in our data. This was also apparent within the polynomial approximation of the fitted Michaelis-Menten model, where the quadratic term contributed minimally to the overall fit, indicating that the underlying trend in the data closely resembled a linear function. Under such conditions, while the relationship was not observed to be strictly proportional, it retains sufficient linearity across the observed range to effectively support estimation of overall shrimp IHHNV loads within ponds, based on qPCR detections from passively sampled eDNA.

The findings of the present study strongly support the utility of passive eDNA sampling as a reliable, rapidly deployable, and non-invasive tool for the detection and quantification of pathogen load in shrimp aquaculture systems, offering a cost-effective approach for pathogen surveillance. Using IHHNV as a model, this study highlights the potential of passive eDNA sampling to enhance the scope of aquaculture biosecurity practices, especially for large-scale, long-term or continuous pathogen monitoring and surveillance programs (Mejías-Molina et al., 2024a). Further refinement is necessary to address the uncertainties in detection dynamics from ponds with lower environmental target pathogen levels, such as those with low host prevalence, low-level host infections, or ponds early in the production cycle, where total infected biomass is low. Similarly, further research is required to appropriately calibrate eDNA signal to host pathogen load across various shrimp life stages (Power et al., 2023; Rusch, 2021), and environmental production conditions including site-specific water chemistry, turbidity, organic load and flow dynamics affecting eDNA persistence, decay and detection (Goldberg et al., 2016; Huerlimann et al., 2020; Kirtane et al., 2020; Wang et al., 2021).

Within the present study, quantitative detection of IHHNV in eDNA was strongly associated with IHHNV load detected in host tissue. However, to a lesser extent, water quality parameters including morning pond pH were observed to have a significant association with eDNA IHHNV detection. Further investigation into the roles of pH, temperature and other water quality variables may help clarify their contributions to IHHNV eDNA dynamics in aquaculture environments. Establishing a standardised approach for sampler deployment and analysis, paired with accurate recording of metadata including water chemistry parameters, is required to facilitate both the enhanced integration of eDNA detection data into farm biosecurity and more robust spatial and temporal analysis (Bessey et al., 2022; Bowers et al., 2021; Zaiko et al., 2018).

4.5. Limitations and considerations in eDNA detection of shrimp pathogens

Molecular surveillance using environmental sampling can achieve higher levels of sensitivity due to the presence of 'legacy' genetic material (extracellular or non-living) (Zaiko et al., 2018). Greater sensitivity may be advantageous for surveillance of pathogens in present, but untargeted, sources such as cohabitating species (Coman et al., 2003; OIE, 2019; Zaiko et al., 2018), which is valuable for assessing pathogen transmission pathways, dynamics, and associated risk within a production system. However, the potential involvement of cohabitating species introduces a critical limitation in interpreting eDNA detection data, as contributions from cohabitating species may confound the

interpretation of infection levels in the aquaculture species. The detection of non-viable pathogens from past infections, or external sources including feed products, must also be considered within an eDNA context (Tacon, 2017; WOAH, 2022). eRNA is more labile than eDNA, with a faster degradation rate that renders it less stable in environmental matrices (Marshall et al., 2021). Due to its rapid production and turnover rate, eRNA can afford an improved capacity to indicate metabolic activity, and by extension, the viability of the detection target (Cristescu, 2019). While the lability of eRNA provides a valuable advantage in some contexts, it currently presents additional challenges for collection and preservation, particularly in field conditions, for non-experts, and in sample processing (Bowers et al., 2021; Kang et al., 2025). With further development of eRNA-based methods, the complementary use of both eDNA and eRNA, particularly through evaluating their relative abundance (e.g., eRNA:eDNA ratios), may enhance interpretation of detection signals and support more robust inference of pathogen viability (Marshall et al., 2021; Pochon et al., 2025).

Within the present study, eDNA detection was compared against detection from host tissue, however, the disconnect between the environmental samples and host organisms remains a persistent challenge for accurately estimating a specific host species' infection level (Bass et al., 2023). Interpretation of pathogen detection from passive eDNA sampling, in the absence of complementary tissue sampling, should be accompanied with consideration of the limitations of environmental sampling, emphasising the appropriate context for eDNA as a system surveillance tool (WOAH, 2022).

For quantitative analysis of endemic pathogens, such as those described in this study, the presence of low level or rare false positive detections are likely to have negligible impact on the results or on-farm decision-making (Goldberg et al., 2016). Within this study, confidence in the true positivity of the eDNA samples was supported by complementary host tissue analysis, confirming the infected host species was present in the environment. While sterile equipment and techniques were utilised meticulously throughout this study, we acknowledge the absence of negative control field samples in our dataset and the potential for eDNA field contamination (Forstchen, 2020). Future field application of eDNA sampling for pathogen surveillance and monitoring should include adequate field negative controls (Forstchen, 2020).

4.6. Future research directions

The work presented in the current study provides statistically supported associations between eDNA detections and confirmed host detections, demonstrating the promise of eDNA for host infection estimation. However, further validation is required, and several knowledge gaps need to be addressed, so that the potential of eNA can be realised and exploited in shrimp aquaculture, and other aquaculture sectors. Foremost, determining the limit of detection from eNA samples in aquaculture systems is required (Childress et al., 2024). Further validation, and efforts to estimate the sensitivity of eNA-based detection as it relates to infection in the aquaculture host species, will be essential for understanding the potential of eNA for early warning detection. However, these efforts will be complicated by site-specific system characteristics (e.g. water quality and other environmental factors; Rusch, 2021; Wang et al., 2021), as well as by potential confounding sources of target signal, such as non-target host species (e.g. cohabitating vector or reservoir species), legacy DNA from past infections, or sources of contamination (e.g. feed), all of which may obscure detection signal of active infections in the target host population within the system (Tacon, 2017; WOAH, 2022). Understanding these interactions and their impacts will be required to understand data-associated caveats and enable biologically relevant interpretation of eNA-based pathogen detection (Bowers et al., 2021). Finally, the development of standardised deployment protocols and metadata recording will be critical for integrating eNA-based pathogen surveillance into farm biosecurity programs and ensuring robust, insightful data is generated.

4.7. Conclusions

The challenges posed by disease in global shrimp aquaculture necessitate rapid and accurate disease management tools. WOAH (2022) recognises eDNA detection as a promising tool in the surveillance of aquatic pathogens. Although further investigation of the eDNA approach is required, the present study demonstrates the promise of passively collected eDNA as a sensitive surveillance tool for indicating IHHNV levels in shrimp hosts, within a commercial aquaculture setting. The method's adaptability suggests its utility for detecting a range of aquatic pathogens beyond IHHNV, and thus, its potential to enhance disease monitoring and biosecurity in aquaculture more broadly.

CRediT authorship contribution statement

Phoebe Arbon: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Maria Andrade Martinez:** Writing – review & editing, Investigation, Data curation. **Tony Charles:** Writing – review & editing, Resources, Investigation, Conceptualization. **Joshua Grima:** Resources, Investigation. **Dean R. Jerry:** Writing – review & editing, Supervision. **Kelly Condon:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

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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Appendix A. Supplementary data

Supplementary material to this article can be found online at https://doi.org/10.1016/j.aquaculture.2025.742709.

Data availability

Data will be made available on request.

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