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Development of eNA Sampling for Early Detection of Pathogens in On-Farm Water Sources

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ABSTRACT

Early detection of livestock pathogens is critical for mitigating risk and implementing timely control or eradication responses. Conventional surveillance methods rely on host sampling, which constrains both spatial and temporal coverage. Environmental nucleic acid (eNA) sampling from livestock water troughs offers a promising alternative for detecting microbial and viral communities associated with livestock health and may facilitate early warning of disease presence. In this study, we evaluated four eNA water sampling methods: syringe, cartridge, funnel and passive filter, for their effectiveness in capturing bacterial and viral eNA communities from livestock troughs. Quantitative PCR was used to assess eNA yield and amplification performance, while 16S rRNA gene metabarcoding and RNA-based viromic (metatranscriptomic) sequencing were used to characterize bacterial and viral community composition and evaluate the effects of sampling method, pore size, and filter material. Filter pore size had the strongest effect on filtered volume but did not significantly influence downstream analysis of microbial and viral community composition. Sampling method and site had the greatest influence on bacterial diversity, whereas viral diversity was influenced by sampling method and RNA concentration. Syringe filters were the most cost-effective method, supporting their suitability for large-scale, industry or landholder led sampling programs. Cartridge filters filtered greater volumes and provided higher richness and may be more suitable for detecting low-abundance targets. Passive filters, which integrate eNA over longer deployment periods, may improve detection of intermittently shed pathogens, though logistical constraints remain. By comparing cost, volume, nucleic acid yield, and community profile, we provide practical guidance for selecting eNA sampling methods to support early pathogen detection at livestock water sources. We recommend that eNA-based surveillance can complement existing diagnostic systems to strengthen biosecurity monitoring.

1 | Introduction

Pathogens, including viral, bacterial, and eukaryotic symbionts capable of causing disease in a host, play an important role in ecosystem function (Frainer et al. 2018). However, pathogens can also pose serious risks to human, animal, plant, and environmental health, as demonstrated by recent outbreaks such as the COVID-19 (coronavirus) human pandemic (Lai et al. 2020) and the ongoing panzootic of highly pathogenic avian influenza (HPAI H5N1) (Charostad et al. 2023). In Australia, several high-risk viral livestock diseases, including foot-and-mouth disease (FMD), lumpy skin disease (LSD), and African swine fever (ASF), are not currently present but remain significant threats. An incursion of any of these pathogens would have profound consequences for animal health, welfare, food production, trade, and the national economy (Buetre et al. 2013).

Early detection, investigation and surveillance are crucial for mitigating such risks and enabling timely control or eradication efforts. Environmental nucleic acid (eNA) methods offer promising alternatives to traditional surveillance (Bass et al. 2015; Amarasiri et al. 2021; Farrell et al. 2021). eNA refers to genetic

material including both DNA (eDNA) and RNA (eRNA) shed by organisms into their environment. Methods using eNA can detect microbial organisms including bacteria and viruses (Ge et al. 2012; Benedicenti et al. 2024), as well as larger macroorganisms (Goldberg et al. 2011; Hinlo, Furlan, et al. 2017; Banchi et al. 2020). Compared with conventional detection methods, eNA based approaches often increase detection sensitivity and efficiency (Dejean et al. 2012).

Traditional surveillance remains essential for determining infection status within individual animals and for confirming active cases of disease. Diagnostic tests such as serology or PCR performed on clinical samples can often provide results within hours to days and remain a key tool, whereas eNA-based approaches, particularly those relying on sequencing, may require additional processing time (Bass et al. 2023). However, eNA analysis offers complementary advantages at the population and environmental level. It enables detection of pathogens independent of host sampling, captures signals from asymptomatic individuals, and supports non-invasive, wide-area surveillance. Integrating conventional diagnostics with eNA-based environmental monitoring can provide a comprehensive and scalable framework for early detection and biosecurity preparedness.

Pathogen eNA has been successfully detected in a wide range of natural and engineered aquatic environments. This includes human pathogens and infectious disease in wastewater (Sims and Kasprzyk-Hordern 2020; Zhang et al. 2022), animal viruses in freshwater (e.g., Ranavirus; Miaud et al. 2019) and marine environments (e.g., salmonid alphavirus; Weli et al. 2020), fungal pathogens such as the amphibian chytrid fungus *Batrachochytrium dendrobatidis* in natural waterways (Schmidt et al. 2013; Brannelly et al. 2020) and aquatic plant pathogens (Menning et al. 2020). In aquaculture eNA has been used effectively to monitor a range of fish pathogens, including viruses, bacteria and parasites (Agawa et al. 2016; Bastos Gomes et al. 2017; Bernhardt et al. 2021; Knoben et al. 2024). High throughput systems can enable the simultaneous detection of multiple pathogens and support routine, high-resolution surveillance in biosecurity. Examples include high throughput qPCR (e.g., major haemorrhagic disease-associated pathogens; Xiong et al. 2026), hybrid Photonic Integrated Circuit–based assays for molecular detection of bacterial aquaculture pathogens (e.g., aquaculture pathogens *Aeromonas salmonicida*, *Vagococcus salmoninarum*, and *Yersinia ruckeri*; Knoben et al. 2024), metabarcoding (e.g., environmental aquatic pathogens; Yang et al. 2025) and metagenomic sequencing (e.g., equine viruses; Blomström et al. 2025).

Applying pathogen eNA monitoring to terrestrial settings requires development of suitable sampling approaches. In agriculture, eDNA has been detected in water sources used during production. For example, irrigation water has been tested for *Leptospira* spp., a zoonotic bacterial pathogen (Gamage et al. 2020), and wash water from produce has been used to detect the invasive brown marmorated stink bug (*Halyomorpha halys*) in agricultural produce (Valentin et al. 2018). Approaches developed for disease detection in aquaculture systems, particularly enclosed or recirculating systems (e.g., Krishna et al. 2026), provide valuable insights into optimal sampling strategies for agricultural surveillance, where water and environmental contact points can similarly serve as reservoirs of pathogen eNA. In large scale livestock systems, as seen in northern Australia, livestock are dispersed across large landscapes, making individual sampling challenging. On-farm water points offer a practical location for eNA surveillance, as animals congregate at these points and may shed DNA/RNA via oral or nasal secretions. Pathogens such as foot-and-mouth disease virus (FMDV) are environmentally stable persisting in soil, water, and surfaces, and can be shed into shared water or feeding sources (Grubman and Baxt 2004). Water troughs thus provide a potential early detection point for herd-level surveillance.

Coupling eNA-based approaches with industry and stakeholder engagement offers opportunities for scalable, cost-effective surveillance. Such participatory monitoring has been successfully implemented in biodiversity assessments and shows promise for biosecurity applications (Zhang et al. 2023). Engaging stakeholders in disease surveillance aligns with national priorities to strengthen Australia's biosecurity system. Although exotic livestock diseases such as FMD and LSD are not present in Australia and thus not targeted directly, our study focuses on developing eNA protocols that can be adopted by industry to enhance early detection capability.

The aims of this study:

1. Evaluate four eNA sampling approaches: syringe filter, cartridge filter, passive filter, and filter funnel for their efficiency in capturing eNA, based on the relationship between water volume filtered and nucleic acid concentration.
2. Assess each sampling method's performance in detecting microbial (bacterial) and viral community composition from livestock water troughs.
3. Identify the most suitable eNA sampling kit for use by industry and landholders, considering ease of use, detection performance, and cost.

2 | Materials and Methods

2.1 | Study Site and Sampling

The Charles Darwin (CDU) Katherine Rural Campus (KRC) (−14.37441° S, 132.15507° E) is located 16 km north of Katherine, Northern Territory, Australia. The KRC covers 4400 ha and includes a cattle stud and agricultural training farm. Water samples for eDNA analysis were collected in November 2023 from two separate paddocks (Sites 1 and 2), each containing a water trough supplied directly and independently by groundwater with no other water access on site (Figure 1). The sites were selected based on the duration of animal occupancy to enable the accumulation of animal-associated microbial and viral communities. Site 1 (Buffalo paddock) contained 50 female Brahman cattle (*Bos indicus*) with calves that had continuous access to the trough for 112 days prior to sampling. Site 2 (Sabi paddock) housed 57 *B. indicus* approximately 6–8 months old that had recently been weaned from their mothers (weaners) that had access to the water trough for 22 days prior to sampling. Site 1 was

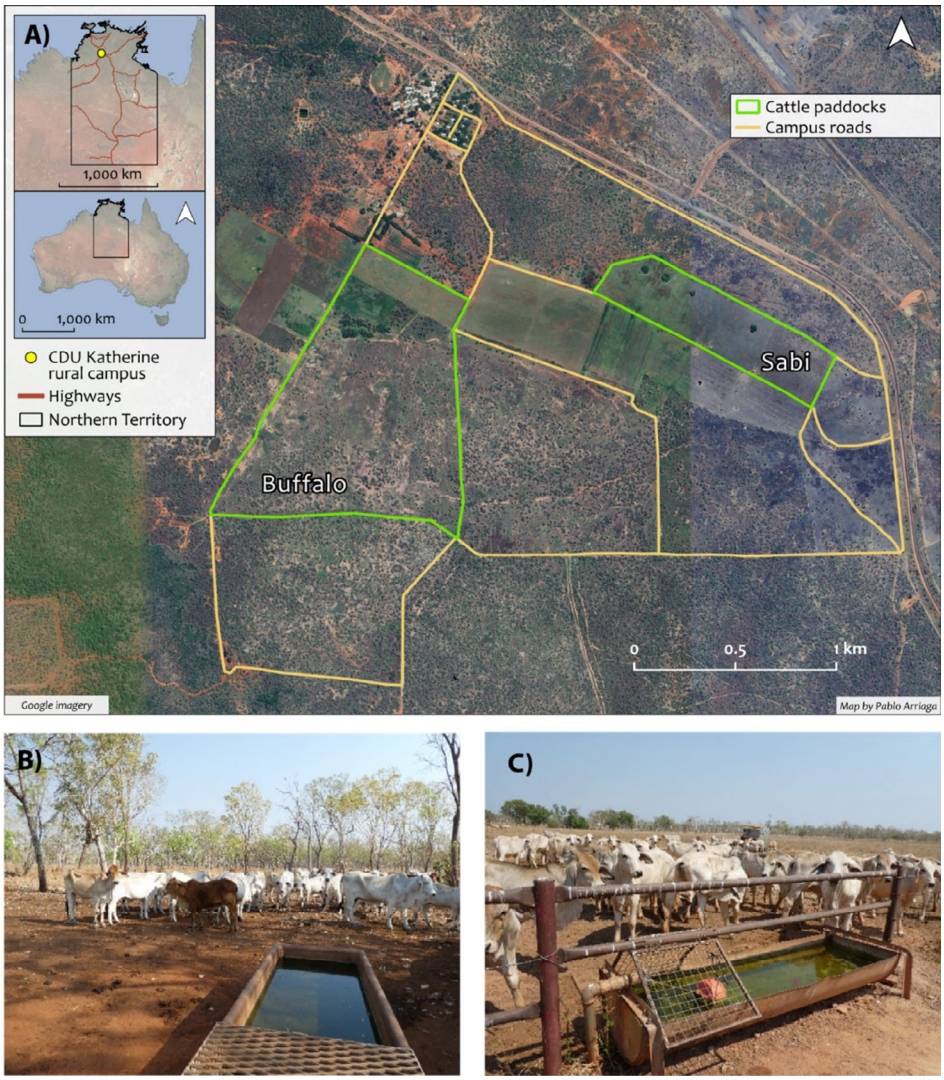

FIGURE 1 | (A) Katherine Research Campus located in the Northern Territory, (B) Site 1 (Buffalo paddock), and (C) Site 2 (Sabi paddock) sampling sites.

shaded by trees for much of the day, providing cooler, lower-light conditions that may favor increased microbial and viral persistence by limiting UV exposure and temperature-dependent degradation processes. In contrast, Site 2 was fully exposed to sunlight, with no surrounding vegetation, resulting in warmer water temperatures and higher light exposure. These two sites provided contrasting conditions in animal use and environmental exposure, enabling evaluation of eNA recovery efficiency and how variation in microbial and viral load influenced nucleic acid recovery and community composition across sampling methods.

2.2 | Pathogen eNA Collection

To evaluate eNA sampling approaches, four eNA capture types were tested and compared: (a) syringe filter, (b) filter cartridge, (c) filter funnel with peristaltic pump, and (d) passive filter (Figure 2, Table 1, Figure S1). Two replicate water samples were collected at Site 1 and Site 2 for each method. The volume of water successfully filtered was recorded for each replicate (Tables S1 and S2).

2.2.1 | Syringe Filter Sampling

Syringe filters are commonly used for citizen science studies due to their portability and ease of use without the need for professional equipment (Zhang et al. 2023). A literature review identified suitable syringe filters (0.22–1.2 μm) available for purchase in Australia. Three syringe filter materials were selected: glass fiber and cellulose acetate (GF + CA), polyethene sulfone (PES) and cellulose acetate (CA), at pore sizes of 0.22, 0.45, 0.8, and 1.2 μm for a total of six syringe filter types (Table 1). A sampling kit was assembled for each filter type comprising a plastic bag, syringe filter, 60 mL syringe, 6 mL syringe pre-filled with 300 μL preservative DNA/RNA Shield (Zymo Research, Irvine, CA, USA), Luer-lock caps, and gloves (Figure S2).

At each site, two independent water replicates were collected for each syringe filter type giving a total of 12 syringe filtered water samples per site. Each syringe filter type was used to filter water until clogging occurred, with the number of syringe refills recorded (Figure S2, Tables S1 and S2). Following filtration, 300 μL of DNA/RNA Shield preservative was added directly into each syringe filter. The syringe filter was sealed with a Luer-lock

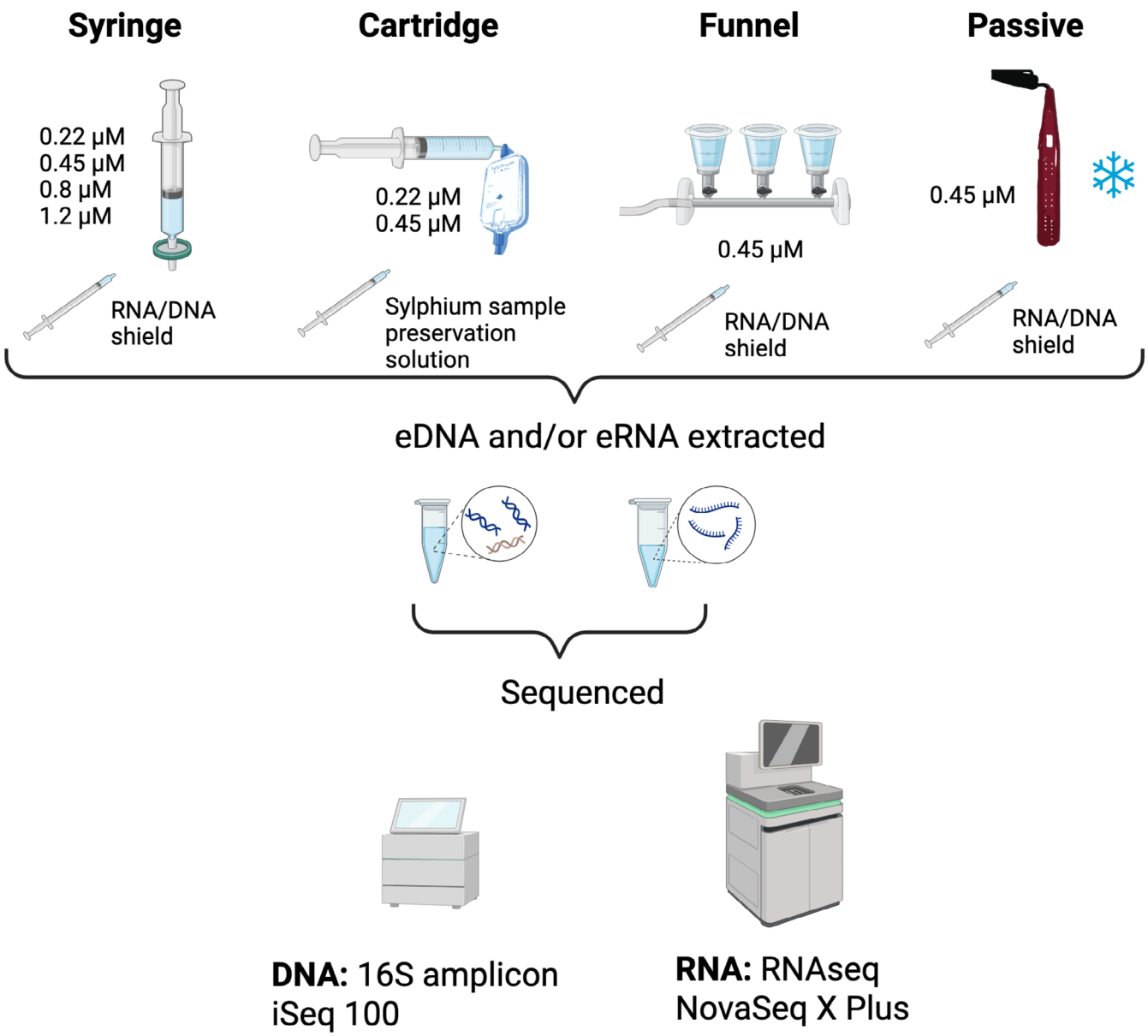

FIGURE 2 | Schematic of eNA capture methods for the detection of pathogens from livestock troughs (created with Biorender, https://biorender.com).

cap, stored on ice and transported back to the Charles Darwin University (CDU) trace DNA laboratory, where it was stored at −20°C prior to extraction. Two negative field controls were processed using 0.8 and 1.2 µm syringe filters by sampling the groundwater directly.

2.2.2 | Filter Cartridge Sampling

Filter cartridges potentially allow larger volumes of water to be processed compared to syringe filters. The Sylphium eDNA Dual Filter Cartridge kits (including capsule with valve connector, 60 mL syringe, 3.5 mL preservative-filled syringe, Luer-lock caps and storage bag) (Figure S3) were used following the manufacturer's instructions (Sylphium Molecular Ecology). At each site, two independent water replicates were collected for each filter cartridge (0.22, 0.45 µm), giving a total of four water samples per site. When filtration was complete, the amount of water filtered was recorded (Tables S1 and S2) and the supplied 3.5 mL of preservative was added, sealed with the Luer-lock caps, and cartridges were stored on ice, transported to CDU, and stored at −20°C prior to extraction. A negative field control was also processed using groundwater for the 0.45 µm filter cartridge.

2.2.3 | Filter Funnel Sampling

Filter funnels, widely used in traditional eDNA workflows, require more extensive equipment including a manifold and pump, and are typically used in laboratory rather than field settings (Piggott 2016; Piggott et al. 2021). Two liters of water were collected from each site and transported to on-site accommodation at KRC. Samples were filtered using a peristaltic pump and 250 mL filter funnels with a pore size of 0.45 µm (Figure 2), with the volume filtered for each replicate recorded (Tables S1 and S2). A total of two water samples per site were filtered. In

TABLE 1 | Types of eDNA sampling approaches used describing filter method type, filter brand, filter pore size, filter material including polyethene sulfone (PES), glass microfiber (GF), cellulose nitrate (CN), cellulose acetate (CA), eNA target, extraction method, and estimated (Est.) cost of sampling kit.

Type	Filter brand	Filter pore size	Filter material	Target	Extraction method	Est. collection cost per sample kit (AUD)[a]
Cartridge	Sylphium eDNA Dual filter	0.22 μm, 0.45 μm	PES	eDNA	Sylphium eDNA extraction kit	$23.00
Syringe filter	Minisart GF	0.2 μm, 0.45 μm	GF + CA	eDNA eRNA	Zymo QuickDNA Miniprep Plus Kit Zymo Quick-DNA/ RNA Viral Kit	$14.00
Syringe filter	Minisart NML Plus	0.8 μm, 1.2 μm	CA	eDNA eRNA	Zymo QuickDNA Miniprep Plus Kit Zymo Quick-DNA/ RNA Viral Kit	$14.00
Syringe filter	Millex	0.22 μm, 0.45 μm	PES	eDNA eNA	Zymo QuickDNA Miniprep Plus Kit Zymo Quick-DNA/ RNA Viral Kit	$13.00
Passive sampler	Torpedo passive sampler	0.45 μm	CN	eDNA eNA	Zymo QuickDNA Miniprep Plus Kit Zymo Quick-DNA/ RNA Viral Kit	$23.00
Filter funnel	Nalgene disposable filter funnel	0.45 μm	CN	eDNA eNA	Zymo QuickDNA Miniprep Plus Kit Zymo Quick-DNA/ RNA Viral Kit	$20.00

[a]Does not include transport or labor costs.

addition, a negative field control was also filtered using groundwater. Filters were placed into sterile DNA Lo-Bind tubes (Eppendorf) containing 300 μL DNA/RNA Shield preservative, stored on ice, transported to CDU, and frozen at −20°C.

2.2.4 | Passive Filter Sampling

Passive filtration, increasingly used in aquatic pathogen detection (e.g., SARS-CoV-2), offers a low-effort, non-mechanized alternative (Bessey et al. 2022; Schang et al. 2021). Two torpedo-style passive samplers, originally developed for wastewater SARS-CoV-2 detection (Schang et al. 2021), were deployed at each site in the water trough for 24 h. The four samplers were then transferred to a cold room, transported in an ice-packed esky to CDU, and stored at −20°C. At CDU, filters were removed from each passive sampler using sterile forceps and placed into sterile tubes containing 300 μL DNA/RNA Shield.

2.3 | Pathogen eNA Extraction

The preservative fluid containing captured biological material was transferred from each syringe filter and cartridge filter to a DNA Lo-Bind tube (Eppendorf) for extraction. Based on previous studies (David et al. 2021), it was assumed the preservative achieved sufficient lysis for downstream processing. For the passive sampler and filter funnel samples, the extractions were performed on the 300 μL DNA/RNA Shield with the retained filters. For the syringe filter, filter funnel, and passive filter samples, one replicate was extracted using the Zymo Quick-DNA Miniprep Plus Kit (Zymo Research) which yielded 50 μL ultra-pure total DNA. The second replicate was extracted using the Zymo Quick-DNA/RNA Viral Kit (Zymo Research) which recovered 50 μL high-quality viral DNA/RNA. DNase 1 (Thermo Fisher Scientific) was applied to the extraction eluate to remove residual DNA prior to downstream RNA-based analyses. The filter cartridge samples were excluded from this analysis as the supplied extraction kit targets DNA and does not recover RNA. Both extraction kits follow a spin column approach and were used following the manufacturer's protocol for samples preserved in DNA/RNA Shield. This design allowed comparison of extraction performance between two nucleic acid extraction kits for each filter type and assessment of how filter material and pore size influenced nucleic acid recovery. The four negative field controls (groundwater) were processed alongside the environmental samples through DNA extraction to detect potential contamination during sampling as well as identify background microbial and viral DNA. For the eight filter cartridge samples, DNA was extracted using

the Sylphium eDNA extraction kit, following the manufacturer's protocol with a final elution of 100 μL (eDNA conservation buffer). A negative template control (NTC) was also included in every batch of eNA extractions.

2.4 | eNA Bacterial Pathogen Analysis—qPCR

Environmental nucleic acid (eNA) quality and quantity were assessed for each sample using both fluorometric and spectrophotometric methods. Sample concentration was measured fluorometrically with the Qubit Fluorometer (Thermo Fisher Scientific, USA), while purity was evaluated using the NanoDrop spectrophotometer (Thermo Fisher Scientific, USA) by recording A260 nm values and A260/A280 nm ratios (Tables S1 and S2). To assess bacterial DNA presence, a single assay targeting the V4 region of the 16S rRNA gene was applied to all samples extracted with the Zymo Quick-DNA Miniprep Plus Kit ($n = 39$). Amplification was performed using primers from the Earth Microbiome Project: 515F (5′-GTGYCAGCMGCCGCGGTAA) and 806R (5′-GGACTACNVGGGTWTCTAAT) (Apprill et al. 2015; Parada et al. 2016). These primers amplify a 290 bp region on the bacterial 16S rRNA gene and are widely used for profiling microbial community composition (Caporaso et al. 2011).

Quantitative PCR (qPCR) was conducted on a Rotor-Gene Q real-time PCR instrument (Qiagen) with the following cycling conditions: initial denaturation at 50°C for 2 min and 95°C for 15 min, followed by 40 cycles of 94°C for 15 s, 50°C for 30 s (annealing), 72°C for 30 s and 80°C for 15 s, followed by a melt curve from 60°C to 95°C with 5°C increments per cycle. Each 20 μL qPCR reaction included: 2× QuantiTect SYBR Green PCR Master Mix (Qiagen), 0.2 μM of each primer, 50 ng/μL bovine serum albumin (ThermoFisher), 8.5 μL of nuclease-free water, and 1 μL of template DNA. Samples were run in duplicate. Six negative controls including the four field sample controls, two extraction controls and a no-template qPCR control were included.

A positive control consisting of genomic DNA from a pure culture of *Vibrio parahaemolyticus* (ATCC 17802, 5.15×10^6 bp, Thermo Fisher Scientific, USA) was included in each qPCR run to verify reagent performance and amplification efficiency. The control DNA concentration was measured using a NanoDrop spectrophotometer. This positive control was not used to generate a standard curve, as the environmental samples contained mixed DNA from multiple bacterial taxa. It was included to assess amplification efficiency and reaction consistency. Cycle threshold (Ct) values from environmental samples were used as relative indicators of bacterial DNA abundance.

2.5 | eNA Bacterial Pathogen Analysis—Metabarcoding

For 16S rRNA gene sequencing, primers targeting the V3-V4 hypervariable regions with Illumina overhang adapter sequences were used for PCR amplification (16SF: =5′ TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG, 16SR: 5′ GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC; Klindworth et al. 2013). Libraries were prepared following the Illumina protocol 16S Metagenomic Library Prep Guide (https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf). The first stage 25 μL PCR contained 2× KAPA HiFi HotStart Ready Mix, 1 μM of each 16S primer and 2.5 μL of template DNA. Amplicons were generated in triplicate for each sample and 5 μL from each replicate was pooled for each sample (total 15 μL) prior to library preparation. The pooled 16S amplicons were purified using AMPure XP beads according to the manufacturer's protocol. The 50 μL Index PCR contained 2× KAPA HiFi HotStart Ready Mix, 5 μL each of Nextera XT Index primers 1 and 2, 10 μL of PCR-grade water and 5 μL of purified pooled amplicon. Indexed PCR products were purified using AMPure XP beads and quantified prior to sequencing. The final pooled equimolar amplicon library (50 pM) was diluted in 10 mM Tris, pH 8.5, to a final concentration of 1 nM for sequencing on the iSeq system. The libraries, comprising 20 pooled indexed samples with a 30% spike-in of Phi X control DNA (Illumina, USA), were loaded onto the iSeq system and sequenced using the iSeq 100 Reagent v2 (300-cycle) cartridge.

Data generated from the iSeq as raw reads in FASTQ format were filtered using the Illumina 16S Metagenomics workflow (Salamon et al. 2022). The high-quality sequences were clustered, and the operational taxonomic units (OTUs) with 99.9% identity were prepared. Sequences were taxonomically classified using the RefSeq RDP 16S v3 database (Alishum 2019) via the high-performance implementation of the Ribosomal Database Project (RDP) classifier (Wang et al. 2007). Sequencing data were rarefied to the depth of the lowest sample, and ASV features with fewer than 10 reads were excluded from downstream analysis.

2.6 | eNA Viral Pathogen Analysis—Whole Genome Sequencing

RNA concentration was quantified using the Qubit RNA High Sensitivity assay kit (Thermo Fisher Scientific, Tables S1 and S2). Quality control (QC) analysis included assessment of RNA concentration (Epoch & Tapestation Bioanalyser), purity based on 260:280 and 260:230 absorbance ratios (Epoch), and RNA integrity metrics including RIN and DV200 values (Tapestation Bioanalyser). Samples that passed the QC were prepared for sequencing using the Illumina Stranded Total RNA RiboZero Plus Microbiome kit at the Ramactiotti Centre for Genomics (University of New South Wales, Sydney). The final pooled libraries were sequenced on a NovaSeq X Plus 10B platform using a 2 × 150 bp paired-end configuration, generating approximately 40 million read pairs per sample. Demultiplexing was performed by the Ramaciotti Centre, and raw Illumina read data was assessed for quality using FastQC (Andrews 2010). Paired-end reads were trimmed using Trimmomatic (v.0.35) (Bolger et al. 2014) with modified parameters: only reads with Phred quality scores > 33 and minimum length > 75 bp were retained for downstream analysis.

De novo assembly of high-quality reads was performed with rnaSPAdes using the --rnaviral option (Bushmanova et al. 2019; Prjibelski et al. 2020). Assembled contigs were

screened for viral origin using ViralVerify (https://github.com/ablab/viralVerify/) before proceeding. Contigs were subsequently aligned against the RNA-dependent RNA polymerase (RdRP) diamond database (v0.90, https://github.com/JustineCharon/RdRp-scan) and the NCBI non-redundant (nr) database (downloaded in August 2023, ftp://ftp.ncbi.nih.gov/blast/db/) using Diamond (Buchfink et al. 2021). The alignment used the '--very-sensitive' mode, with a minimum ORF length of 100 bp (--min-orf 100), and an E-value threshold of 1e-5 (−e 1e−5), with output in DIAMOND DAA format (f −100). Resulting DAA files were processed (Meganized) in MEGAN6 using taxonomic classification based on the RdRP and nr databases (Bağcı et al. 2021). Classification outcomes were explored using the comparison and clustering tools within MEGAN. Raw sequencing data for this study are available from the NCBI Sequence Read Archive under accession numbers SRR33701257–SRR33701294 under BioProject PRJNA1268115. Viral contigs were also cross-checked against the Australian Biosecurity Genomic Database for viruses of terrestrial animals (https://github.com/ausbiopathgenDB/AustralianBiosecurityGenomicDatabase).

2.7 | Statistical Analysis

We analyzed the effects of filter pore size, filter material, filter type, and site on filtered water volume, total DNA and RNA concentrations, and Ct values for bacterial qPCR detection. Due to the non-normal distribution of filtered volume and total DNA/RNA concentration, as confirmed by Shapiro–Wilk tests ($p < 0.001$) and skewness analysis, general linear models (GLMs) with a Gamma distribution and log-link function were applied. Filtered volume was modeled as a function of filter pore size, filter material, filter type, and site. For total DNA and total RNA concentration, filter pore size, filter material, filter type, volume, and site were included as predictors.

All categorical variables (filter material, filter type, site) were treated as factors, while filter pore size and volume were treated as continuous variables. Variance inflation factors (VIFs) were used to assess collinearity among predictors prior to modeling. The maximum VIF values, 1.217 (volume), 1.015 (total DNA), and 2.94 (total RNA), were all below the commonly accepted threshold of 5, indicating that multicollinearity did not significantly influence model results. Model selection was based on Akaike's information criterion (AIC) and non-significant predictors where necessary. Significance of individual predictors was assessed using analysis of variance (ANOVA).

Differences in bacterial Ct values across filter types and pore sizes were evaluated using linear regression models and Shapiro–Wilk tests for normality. Alpha diversity metrics (Shannon index and Richness) were calculated for bacteria (genus level) and viruses (family level) using taxonomically assigned reads and compared among groups using Kruskal–Wallis tests. Beta diversity was assessed using Bray–Curtis dissimilarity and visualized using non-metric multidimensional scaling (NMDS) via the metaMDS function in the vegan package (Dixon 2003). Community-level differences were tested using permutational multivariate analysis of variance (PERMANOVA) with 9999 permutations (adonis2 function in vegan).

All statistical analyses were conducted in R v3.9.4 (R Core Team 2024) using the lme4 (Bates et al. 2015), MuMIn (Bartoń 2024), and emmeans (Lenth 2025) for GLM analyses, lm() for regression, and vegan (Oksanen et al. 2025) packages for diversity analyses. Data visualizations were produced using ggplot2 (Wickham 2016) and MEGAN 7 (Bağcı et al. 2021). Final figure panels were arranged using Adobe Illustrator.

3 | Results

3.1 | Effect of Sampling Approach on Volume and eNA Concentration

The best fitting GLM for predicting filtered water volume included filter pore size, filter type, filter material, and site as predictors (Table S3). Filter pore size had the strongest positive

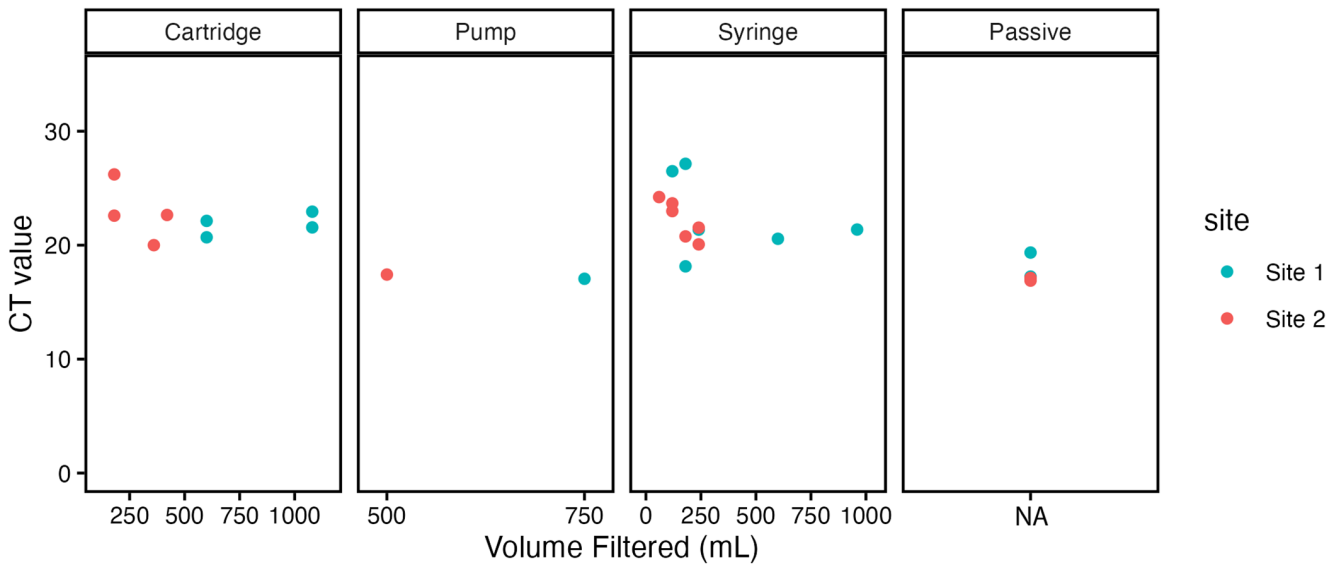

FIGURE 3 | Relationship between qPCR Ct values and volume filtered for each sample method for Site 1 (orange) and Site 2 (blue).

effect on filtered volume ($p < 0.001$), indicating that filters with larger pore sizes significantly increased the volume of water processed. Filter type also had an effect, with syringe filters yielding significantly lower volumes compared to filter funnels ($p < 0.001$). Among filter materials, CA showed a moderate but significant effect ($p = 0.049$), whereas other materials were not significant. Site was a highly significant predictor ($p < 0.001$), with lower filtration efficiency observed at Site 2 compared to Site 1. ANOVA results supported the importance of filter type, filter pore size, and site (all $p < 0.001$) as the most influential predictors of filtered volume. Filter material contributed a smaller, yet statistically significant effect ($p = 0.006$).

In comparison, the best fitting GLM for predicting total DNA concentration included filter type, filter pore size, and filter material, while site and filtered volume were not retained in the final model (AIC = 156.3) (Table S4). Filter type had a significant effect, with syringe filters yielding lower DNA concentration compared to filter funnels ($\beta = 0.8126$, $p = 0.035$). Although filter size was positively correlated with DNA concentration, the relationship was not statistically significant ($\beta = 1.5665$, $p = 0.125$). Filter material had no significant individual effect on DNA concentration ($p > 0.05$). However, ANOVA results indicated that both filter type and filter pore size were significant predictors of DNA concentration ($p < 0.001$), while filter material had a weaker but significant effect ($p = 0.027$). For total RNA concentration, the final GLM identified filter material CN as having a moderate but statistically significant effect ($p = 0.021$; Table S5).

3.2 | Comparison of Amplification of Bacterial DNA Between Capture Approach

All samples successfully amplified in the 16S rRNA gene qPCR assay, including the positive control, while no amplification was detected in the negative controls. The lowest Ct values, indicative of higher bacterial eDNA concentrations, were observed in samples collected using the filter funnel and passive sample approach (Figure 3). The distribution of Ct values was confirmed to be normal on the Shapiro–Wilk test ($W > 0.952$, $p = 0.262$). Ct values did not significantly vary as a function of the volume of water filtered (ANOVA: $R^2 = 1.37 \times 10^{-5}$, $F_{1,24} = 0.0003$, $p = 0.986$), suggesting that the sampling method, rather than the volume processed, had a greater influence on bacterial eDNA detection efficiency (Table S6).

3.3 | Sampling Method Influences Bacterial and Viral Community Diversity

The eNA Bacterial Pathogen run yielded 4,682,754 FASTQ reads in total and resulted in a total of 2,341,377 16S rRNA bacterial hits and 2,098,366 RNA-seq hits across all sampling methods. Bacterial community richness and Shannon diversity were moderately higher in samples collected with cartridge filters compared to other filter types (Kruskal–Wallis test, $p = 0.046$) (Figure 4A,B). Non-metric multidimensional scaling (NMDS) based on Bray–Curtis dissimilarity of taxonomically assigned bacterial genera revealed distinct clustering by site (PERMANOVA, $p = 9.99 \times 10^{-5}$), with passive filter samples clustering more closely to each other than to samples from their respective sites (Figure 4C,D, Table S7). This suggests both site and sampling approach influence bacterial community composition.

Taxonomic assignment of RNA-seq data using the nr and RdRP databases identified 23 viral families with variable abundance across samples (Table S8). Viral family richness did not significantly differ between filter types at Site 1 ($p = 0.191$) but was moderately significant at Site 2 ($p = 0.042$; Figure 5A). At Site 1, the two passive filters showed the lowest viral family richness with 7 and 8 viral families detected respectively. Shannon diversity values for viral communities were generally low (< 2) across both sites (Figure 5B). Viral community composition was significantly influenced by filter type ($p = 0.0019$; Figure 5C,D) and total RNA concentration ($p = 0.034$).

The most abundant viral family was *Mimiviridae*, comprising 12.1% and 10.2% of all viral hits at Site 2 and Site 1, respectively. Several viral families detected in our samples, including *Birnaviridae*, *Coronaviridae*, *Orthomyxoviridae*, *Phenuiviridae*, and *Sedoreoviridae*, were also listed in the Australian Biosecurity Genomic Database for viruses of terrestrial animals. Additionally, RNA-seq hits assigned to Eukaryota accounted for 43, 893 contigs, Bacteria for 33, 230 contigs and Archaea for 1, 503 contigs of the total cellular-organism hits across all samples.

3.4 | Comparison of Cost

The estimated per-sample cost of each eNA sampling method, including nucleic acid extraction, is summarized in Table 1. Syringe filter kits were the most cost-effective, with a consistent cost of approximately $13–$14 per sample across different pore sizes and materials. In contrast, the other sampling methods, cartridge filters, filter funnels, and passive filters, each exceeded $20.00 per sample.

4 | Discussion

This study evaluated four eNA sampling approaches for detecting bacterial and viral communities in livestock troughs and assessed their suitability for practical on-farm pathogen surveillance. All sampling methods successfully captured bacterial and viral eNA, enabling community profiling and demonstrating that trough water can serve as an effective environmental reservoir for early-warning surveillance in livestock systems. We have shown that eNA approaches for trough water can detect pathogen-associated signatures that may signal elevated disease risk. The key differences among methods based on cost, contamination risk, filtration efficiency, and community richness provide important considerations for their application in routine or targeted on-farm biosecurity monitoring. Based on cost, ease of use, and enclosed design, syringe filters are likely to be the easiest to implement in an industry or stakeholder led surveillance. However, other approaches that can process greater volumes may improve detection of pathogens that occur at low abundance. Passive samplers, which captured distinct microbial profiles, may integrate eNA signal over a longer time window, increasing the likelihood of detecting intermittently shed pathogens.

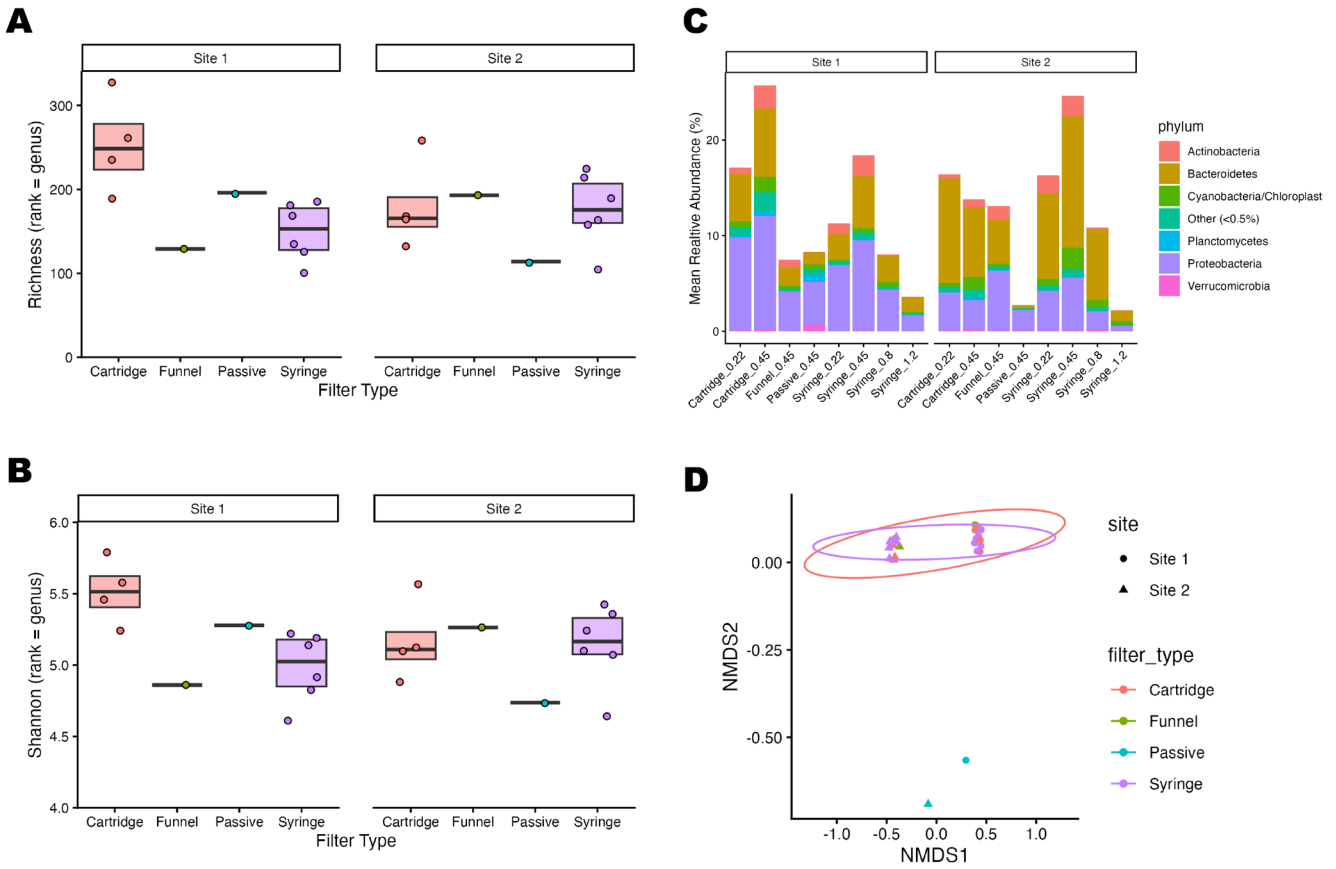

FIGURE 4 | Alpha and beta diversity of bacterial communities detected using eNA metabarcoding across sites and filter type: (A) Bacterial genus richness. (B) Shannon diversity index. (C) Relative abundance of bacterial communities (D) Non-metric multidimensional scaling (NMDS) ordination of bacterial community composition. The ordinal ellipses represent the 95% confidence interval of the standard error.

Filter pore size was the most important predictor of filtration volume, with larger pore sizes significantly increasing throughput. This finding aligns with previous studies showing that smaller pore sizes can increase resistance, leading to clogging and reduced filtration efficiency (Liu et al. 2024). Increased filtration volume is relevant for pathogen surveillance because filtration efficiency determines the amount of water processed and therefore the probability of capturing low-abundance pathogens. While other studies have reduced DNA yield with larger pore sizes (Eichmiller et al. 2016; Liu et al. 2024), we did not observe this in our study. Sampling approach also significantly affected filtered volume, with syringe filters yielding lower volumes than filter funnels. This could be due to greater resistance, smaller surface area, and the need for manual pressure, which we observed became increasingly difficult during filtration using syringe filters. Site-specific differences were also significant, with reduced filtration efficiency at Site 2 likely due to water turbidity and algal presence, compared to the clearer water at Site 1. Filters with larger pore sizes have been found to perform better under turbid conditions (Robson et al. 2016). Site-specific differences suggest environmental conditions such as turbidity can constrain filtration efficiency and should be considered when designing surveillance protocols. Filter material had a smaller effect, with CA filters associated with increased filtration volume. However, this result may also be influenced by larger pore size as this material was used in the syringe filters with 0.8 and 1.2 μm pore size.

In contrast, total DNA and RNA concentrations were more strongly influenced by filter chemistry rather than by filtration volume. Filter type had a significant effect on DNA concentration, with syringe filters yielding less DNA than filter funnels. CN filters yielded significantly higher RNA yields, indicating that filter material choice can enhance viral eNA recovery, which is a critical factor for viral pathogen surveillance. This agrees with other studies on the higher filtration performance of CN filters over other materials such as PES (Liang and Keeley 2013; Renshaw et al. 2014). CN was used in both the passive samplers and filter funnel methods. Our results suggest that while filtration volume is driven by physical and environmental constraints, DNA and RNA yield may depend more on filter material chemistry and capture efficiency. As total DNA/RNA recovery was not strongly driven by volume filtered, this suggests that filter chemistry and handling need consideration, rather than maximum throughput alone.

Although total DNA concentration is often used as a proxy for eNA capture efficiency, it does not always reflect the abundance of the target organism. qPCR-based quantification offers a more specific and functional assessment of method performance (Eichmiller et al. 2016; Hinlo, Gleeson, et al. 2017; Spens et al. 2017). In our study, total DNA concentration was strongly correlated with bacterial qPCR amplification. While our qPCR targeted a general bacterial 16S rRNA sequences rather than specific pathogens, this relationship suggests that methods that

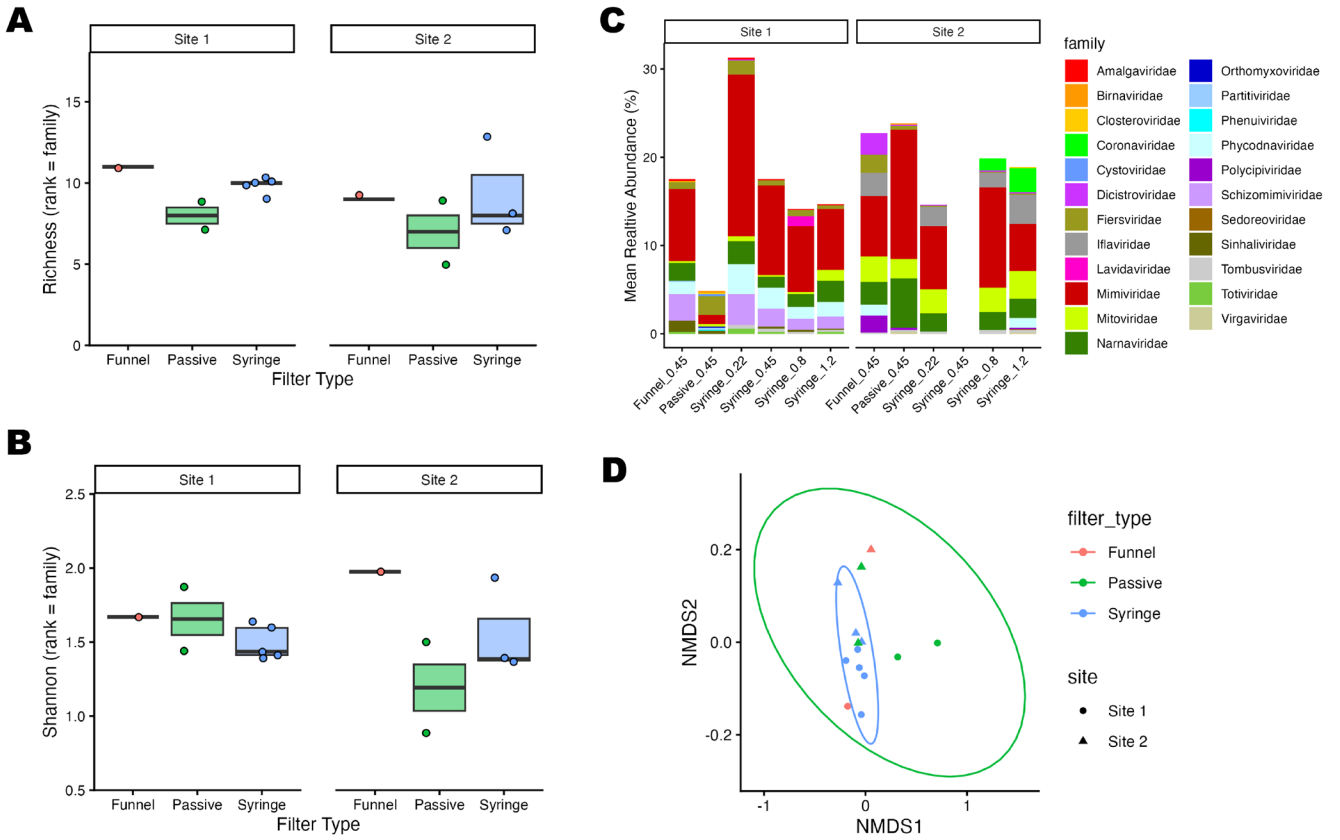

FIGURE 5 | Alpha and beta diversity of viral communities detected using metatranscriptomic (RNA-based) virome sequencing across sites and filter type: (A) Viral family richness. (B) Shannon diversity index (C) Relative abundance of viral taxa. (D) NMDS ordination of viral community composition. The ordinal ellipses represent the 95% confidence interval of the standard error.

maximize DNA yield such as the filter cartridge will enhance downstream pathogen-targeted detection.

Community diversity analyses showed that bacterial richness and Shannon diversity were moderately higher in samples collected using filter cartridges, and bacterial community composition differed significantly by site. NMDS ordination indicated that passive filter replicates were more similar to each other than to other methods at the same site, suggesting method-dependent community capture. Other studies have shown that CN filters (as used in the passive samplers) yielded the most eDNA and consistent community composition (Liang and Keeley 2013; Majaneva et al. 2018). Viral community analysis detected 23 viral families, including *Birnaviridae*, *Coronaviridae*, *Orthomyxoviridae*, *Phenuiviridae*, and *Sedoreoviridae* listed in the Australian Biosecurity Genomic Database. *Mimiviridae*, a large and diverse family of eukaryotic viruses found in aquatic environments (Claverie and Abergel 2018), was the most abundant viral family across both sites. While family-level assignments cannot diagnose disease, they provide an important early warning that could trigger targeted qPCR assays for specific pathogens of concern. Our RNA-seq results detected sequences from all three cellular domains of life (Eukaryota, Bacteria and Archaea), demonstrating the broad surveillance potential of eNA for viral, eukaryote, microbial and archaeal pathogens in environmental samples.

From an end-user perspective, practical considerations distinguish the four sampling approaches. Syringe filters were the most cost-effective, enclosed and simple to deploy with minimal equipment and would be suitable for frequent or large-scale surveillance, as seen in citizen science applications (Burian et al. 2023; Knudsen et al. 2023; Zhang et al. 2023). While filter funnels processed greater volumes and captured more DNA, they require open handling and longer filtration times (e.g., Andruszkiewicz et al. 2017) which increases the contamination risk so are better suited to controlled or laboratory settings. Passive filters required minimal equipment, potentially detect temporal signal and may be well-suited to remote deployment. However, reliance on cold-chain transport especially for RNA limits field practicality. Filter cartridges are also promising as an alternative to syringe filters, although more costly, as they had the highest richness and nucleic acid yield, filtered larger volumes so may be the best choice when targeting low-abundance or emerging pathogens, or when higher sensitivity is required.

Citizen science approaches have primarily been applied to biodiversity monitoring, but examples show potential for invasive species and pathogen detection (Miralles et al. 2016; Gysin et al. 2022; Nielsen et al. 2024). The lower cost and ease of use of syringe filter kits could support their application in large-scale or community-led sampling efforts. Alternatively, at a higher cost, the filter cartridge approach was easy to use and showed promising results for bacterial richness. While these methods can be simplified for end users, data quality is dependent on correct implementation and handling.

A limitation of this study is the absence of confirmed pathogen cases at the study sites. Therefore, method performance was not

evaluated against known disease targets. Specific pathogens, such as liver flukes *Fasciola* spp., are best detected via qPCR assays (Rathinasamy et al. 2021), whereas viromic and bacterial community analyses are better suited for broad-scale screening and early warning. As demonstrated by Duval et al. (2024), eDNA can effectively monitor the presence of pathogens such as *Tetracapsuloides bryosalmonaei*, a pathogen of *Salmo trutta*, but presence alone may not indicate infection. Studies have emphasized that eNA detection indicates potential exposure or infection, but definitive diagnosis still requires host tissue analysis (Bass et al. 2023).

We conducted two replicates per method at each site, one for bacterial analysis and one for viromic profiling, and additional replication would improve confidence in results. While spatial variation may be limited in small systems like water troughs, temporal variation remains important (Dejean et al. 2012; Baselga et al. 2013; Beentjes et al. 2019). Replication is also crucial for detecting rare taxa (Grey et al. 2018). Though multiple replicates may be challenging in citizen science contexts, the benefits of broader spatial and stakeholder engagement may outweigh logistical constraints. A standardized eNA sampling protocol underpinned by statistical design will enhance surveillance potential at water troughs and similar systems.

Our study supports that the trough environment or other shared drinking or feeding environments could represent high-value sentinel locations for pathogen surveillance, particularly in extensive grazing systems where direct animal sampling is impractical. To improve resolution to species-level differences will require careful consideration of marker choice as many regions used are suitable for community taxonomic profiling but unable to assign the sequence to genus and below (Bass et al. 2023). Water troughs in open environments also offer a unique interface for wildlife-livestock interactions. In similar contexts, waterholes have been identified as hotspots for microbial and pathogen exchange wildlife-livestock interface (Barasona et al. 2017; Herrero-García et al. 2024). For example, in semiarid Mediterranean habitats of the Iberian Peninsula, waterholes represent a hotspot for the maintenance and cross-species transmission of members of the *Mycobacterium tuberculosis* complex causing animal tuberculosis (Barasona et al. 2017; Pereira et al. 2023). In our study, water troughs and man-made dams are the only water sources available on-site and are frequented by non-target wildlife, increasing the likelihood of cross-species eNA signatures.

Ultimately, while eNA methods offer powerful tools for pathogen risk identification, they should complement rather than replace existing surveillance and diagnostic systems. eNA from livestock troughs reflects environmental presence, not infection status, and may detect pathogens shed by wildlife or transient individuals. However, by identifying pathogen families or shifts in viral or bacterial community composition, eNA offers a scalable tool for early risk detection in extensive livestock systems where individual testing is challenging. False positives from eNA alone could lead to severe regulatory and economic consequences. Therefore, robust protocols, quality control, and confirmatory testing remain essential. This study provides foundational evidence supporting the use of eNA monitoring in livestock water sources and offers insights into practical, scalable sampling strategies suitable for early warning and pathogen surveillance frameworks.

Author Contributions

M.P.P. contributed to project conception and design. M.P.P. and A.M. contributed to the acquisition and analysis of data and writing of the manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are openly available in NCBI Sequence Read Archive at https://www.ncbi.nlm.nih.gov/bioproject, reference number PRJNA126811, with individual run accession numbers SRR33701257–SRR33701294.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** The four different filtration methods tested in the study, including (A) syringe filters (Minisart: 0.2, 0.45, 0.8, and 1.2 μm), (B) Sylphium eDNA Dual filter (0.45 μm), (C) Nalgene disposable filter funnel (0.45 μm), and (D) Torpedo passive sampler (0.45 μm). **Figure S2:** Sampling kit comprising a plastic bag, syringe filter, 60 mL syringe, 6 mL syringe pre-filled with 300 μL preservative DNA/RNA Shield (Zymo Research), Luer-lock caps, and gloves. **Figure S3:** Sylphium eDNA Dual Filter Cartridge sampling kit comprising a plastic bag, capsule filter, with valve connector 60 mL syringe, 6 mL syringe pre-filled with 350 μL preservative (supplied), Luer-lock caps. **Table S1:** Types of eDNA sampling approaches used describing filter method type, filter brand, filter pore size, filter material including polyethene sulfone (PES), glass microfiber (GF), cellulose nitrate (CN), cellulose acetate (CA), volume filtered (vol. (mL)), extraction method, and DNA concentration (ng/μL) collected from Site 1 (Buffalo paddock). **Table S2:** Types of eDNA sampling approaches used describing filter method type, filter brand, filter pore size, filter material including polyethene sulfone (PES), glass microfiber (GF), cellulose nitrate (CN), cellulose acetate (CA), volume filtered, extraction method, and DNA concentration (ng/μL) for samples collected from Site 2 (Sabi paddock). **Table S3:** Parameter coefficients and *p* values for the full and best GLMs for the relationships between volume and four parameters (filter material, filter type, site, and filter pore size). *p* values in bold are significant. **Table S4:** Parameter coefficients and *p* values for the full and best GLMs for the relationships between total DNA concentration and five parameters (volume, filter material, filter type, site, and filter pore size). *p* values in bold are significant. **Table S5:** Parameter coefficients and *p* values for the full and best GLMs for the relationships between total RNA concentration and five parameters (volume, filter material, filter type, site, and filter pore size). *p* values in bold are significant. **Table S6:** Parameter coefficients and *p* values for the full and best GLMs for the relationships between qPCR cycle threshold (ct) and six parameters (volume, filter material, filter type, site, filter pore size, and total DNA concentration). *p* values in bold are significant. **Table S7:** Bacteria sequence counts per genus for each method and site showing replicate used, filter size, filter material, volume filtered, and DNA concentration. **Table S8:** Viral sequence counts per family for each method and site showing replicate used, filter size, filter material, volume filtered, and RNA and DNA concentration.