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RESEARCH

Exploring the Use of Passive Samplers for the Surveillance of Avian Influenza Viruses in Wetlands: A Laboratory and Field Validation Study

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Abstract

Surveillance in wild birds is essential for the timely detection of high pathogenicity avian influenza (HPAI) strains. As flocks congregate in large numbers in wetlands and may potentially contaminate the environment with pathogens, the monitoring of such water bodies represents an attractive opportunity to complement animal testing and to improve surveillance for avian influenza. To increase sensitivity, water concentration is often required but available methods based on (ultra)filtration and precipitation are mostly limited by the use of pumping equipment and by the need to identify the representative sample volumes. In contrast, passive samplers (PS) offer a cost-effective and scalable solution that requires basic devices for the deployment of adsorbent materials and minimal training for their installation in the field. This study evaluated nine materials for their virus adsorption efficiency in brackish and freshwater. Cotton gauze, nitrocellulose, and nylon showed the best performance across different deployment times, with the highest recovery after 24 h. Shorter (3 h) and longer (7 days) deployments also proved effective, accommodating different sampling regimens according to the logistical needs. Importantly, PS revealed their efficacy in adsorbing also deteriorated virions or in dynamic ecosystems subjected to changes in water volumes. Field trials in wetlands corroborated laboratory findings and demonstrated that PS allowed detecting avian influenza virus (AIV, including HPAI strains) genome in water bodies, yielding consistent results with active surveillance in wild birds. By offering a simple, cost-effective, and versatile solution, PS represent a promising tool for environmental AI monitoring and can successfully complement existing avian influenza surveillance activities.

Keywords Avian influenza virus · Viral RNA · Passive samplers · Environmental monitoring · Molecular water testing · Wetlands

Introduction

Avian influenza (AI) is a highly contagious disease of birds caused by type A Influenza viruses (AIVs) of the *Orthomyxoviridae* family. Based on the antigenic properties of the hemagglutinin (HA) and neuroaminidase (NA) surface proteins, influenza viruses affecting birds are currently divided into 16 HA and 9 NA subtypes, which are typically referred to as low pathogenicity avian influenza viruses (LPAIV) (Capua & Alexander, 2009). Only the H5 and H7 subtypes have demonstrated the ability to mutate into high pathogenicity viruses (HPAIV) by acquiring multibasic amino acids at the cleavage site of the HA, which results in a fatal systemic infection in poultry (Banks et al., 2001; Duan et al., 2007; Horimoto et al., 1995).

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Wild waterfowl and sea birds belonging to the *Anseriformes* and *Charadriiformes* orders serve as natural reservoir for LPAIV (Olsen et al., 2006; Stallknecht & Shane, 1988; Webster et al., 1992). However, in 2005, these migratory birds were also held responsible for disseminating high pathogenicity H5 viruses of the goose/Guangdong lineage (Gs/GD) from central China to Europe and Africa (Chen et al., 2006). This laid the foundations for subsequent reassortments with LPAIV and genetic diversification into clades and subclades (Smith & Donis, 2015). Of these, clade 2.3.4.4b has undoubtedly been dominating the scene since 2016, initiating a series of recurrent epizootics in Asia, Africa, and Europe in both wild and domestic birds (Fusaro et al., 2019; Lee et al., 2017; Lycett et al., 2020; Verhagen et al., 2021) that resulted in its establishment within the wild European avifauna (Pohlmann et al., 2022). Soon after, the endemicity of 2.3.4.4b viruses in wild migratory birds favored their dissemination also to North America, South America, and Antarctica, which caused an unprecedented panzootic with significant implications for the global poultry industry, wild animal conservation, and public health (Banyard et al., 2024; EFSA, 2024a; Erdelyan et al., 2024; Kandeil et al., 2023; Koopmans et al., 2024; Ruiz-Saenz et al., 2023).

Such a shift in AI ecology underscores the need for an enhanced surveillance of wild birds and a more proactive approach to promptly implement control measures in response to new viral incursions (EFSA, 2024b). As water bodies act as sustenance, shelter and breeding grounds for most migratory birds, these habitats represent relevant hotspots for virus maintenance and deserve to be considered as supplementary targets to animal samples to increase the sensitivity of surveillance. Indeed, water specimens can circumvent representativeness limitations associated with the sampling of target bird species and offer insights into the composition of AIV in wild avifauna at a broader population level (Pepin et al., 2019). This appears particularly relevant in ecosystems with low-density or highly transient bird populations, where the likelihood of capturing a sufficient number of birds during sampling events may be low. One of the prerequisites for considering water as an eligible matrix for surveillance is its recognized role in bird-to-bird transmission via the fecal–oral route (Ahrens et al., 2022; Pepin et al., 2023; Roche et al., 2009; Zhang et al., 2022). Recent studies have demonstrated that the adaptation of H5 HPAIV of clade 2.3.4.4b to ducks results in efficient shedding through feces, which further increases the interest in water as a substrate for surveillance (Beerens et al., 2021; Filaire et al., 2024; James et al., 2023). Another crucial factor that facilitates the detection of AIV in the environment is the high viral persistence observed in natural water sources. This has been extensively demonstrated in laboratory settings under field-simulated conditions and diverse environmental parameters (Keeler et al., 2013, 2014; Nazir et al., 2010; Nielsen et al., 2013; Ramey et al., 2022).

These evidences have called for more research studies based on water sampling and testing to monitor AI in wetlands and at the wild/domestic interface. A recent systematic review of the presence of the influenza virus in different environments, including wild bird habitats, has revealed a considerable range in positivity rates (0.4–69.8%) (Kenmoe et al., 2024). This variability may partially be attributed to differences in the sensitivity of the analytical methods employed for laboratory testing. Indeed, one of the challenges of an efficient environmental surveillance is represented by the high dilution of the virus in water, which needs a pre-analytical enrichment. The most common strategies comprise single or in-series filtration (including ultrafiltration) for large water volumes (Ahrens et al., 2023; Hubbard et al., 2023; McCuen et al., 2021), precipitation, filtration, or adsorption with red blood cells for small volumes (Khalil et al., 2022; Lickfett et al., 2018; Okuya et al., 2024; Rönnqvist et al., 2012; Zhang et al., 2022), or a combination of these techniques (Deboosere et al., 2011; Germeraad et al., 2020; Horm et al., 2012). If on one hand these methods proved effective and allowed AIV detection and typing from water samples, on the other sampling large volumes proved rather challenging in terms of collection, preservation, and transportation to the laboratory. Additionally, the availability of pumping equipment for on-site filtration may be a limiting factor. Conversely, the use of minimal water volumes is constrained by their inadequate representation of the entire water body.

Passive samplers (PS) are a promising alternative to active concentration systems as they can be easily deployed on-site at the point of need, and do not require trained personnel nor the use of sophisticated equipment. Given their usability and cost-effectiveness, PS attract considerable and increasing attention from the scientific community, and their recent application for the surveillance of SARS-CoV-2, fecal bacteria, and other respiratory viruses in urban wastewater, including Influenza A and B, has demonstrated their effectiveness (Farkas et al., 2024; Geissler et al., 2024; Hayes et al., 2021; Li et al., 2022; Mejías-Molina et al., 2023; Schang et al., 2021). Nevertheless, their suitability for monitoring the environmental prevalence of AIVs in wetlands has yet to be determined. In this study, we conducted an extensive laboratory validation using natural brackish or freshwater spiked with AIV to (i) assess the adsorbent capacity of different materials, (ii) optimize laboratory protocols for virus elution, and (iii) evaluate the effect of deployment time on the effectiveness of passive samplers. Finally, we evaluated the usability of PS in a wetland located along the migratory routes of waterfowl by comparing their ability to detect AIV genome to that of swabs collected from captured wild birds during active surveillance campaigns in the same site.

Materials and Methods

Virus Propagation and Quantification

The H10N1 strain A/mallard/Italy/4518/2007 (Bonfante et al., 2014) was propagated in 9–11-day-old embryonated SPF chicken eggs. The virus load in the allantoic fluid was ascertained through a digital droplet RT-PCR assay (RT-ddPCR) and expressed as genome copies (GC). The amplification reaction was set up using the One-Step RT-ddPCR Advanced Kit for Probes (Bio-Rad Laboratories, Hercules, CA, USA), 5 µl RNA, 0.9 µM primer for, 0.22 µM each primer rev and 0.25 µM probe targeting the AIV matrix (M) gene (Heine et al., 2015), and nuclease-free water up to 22 µl. Droplets were generated using a QX200 Droplet Generator (Bio-Rad Laboratories, Hercules, CA, USA), in accordance with the manufacturer's instructions. The amplification was conducted on a C1000 Touch Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) at the following thermal cycling conditions: 45 °C for 60 min, 95 °C for 10 min, 50 cycles of 95 °C for 30 sec (ramp rate 2 °C/sec) and 58 °C for 1 min (ramp rate 2 °C/sec), and 98 °C for 10 min. The positive and negative partitions were scanned with the QX200 Droplet Reader (Bio-Rad Laboratories, Hercules, CA, USA) and data were analyzed using the QX Manager Software Standard Edition, Version 1.2 (Bio-Rad Laboratories, Hercules, CA, USA).

Virus Spiking of Water Samples

To evaluate the adsorbent capacity of the different materials under realistic usage scenarios, we sampled natural freshwater and brackish water specimens from wetlands located in north-eastern Italy to prepare virus spiked. The freshwater sample was collected from a resurgence lake in San Giovanni Lupatoto (Verona) and found to have a pH of 7, a salinity of 0 ‰, and NO_2 and NO_3 concentrations of 0 ppm. Brackish water was obtained from Valle Figheri (Venice), exhibiting a pH of 7, a salinity of 5 ‰, 2 ppm of NO_2, and 0 ppm of NO_3. The water samples were gathered in May 2024, at a time when no AI outbreaks were reported in the area (EURL Avian Flu Data Portal 2024). The absence of AIV in the samples was further confirmed by subjecting 50 ml water aliquots to polyethylene glycol (PEG 8000) precipitation according to the methodology described in La Rosa et al. (2021), followed by real-time reverse transcription polymerase chain reaction (qRT-PCR) targeting AIV M gene (see Sect. "Preliminary selection of adsorbent materials and laboratory protocols"). In all the experiments, except when otherwise specified, water samples were contaminated with the H10N1 strain to obtain a final concentration of 10^8 GC per liter (GC/L), corresponding to approximately 30 quantification cycles (Cq) by qRT-PCR.

Preliminary Selection of Adsorbent Materials and Laboratory Protocols

Nine different adsorbent materials selected from previous studies (Farkas et al., 2024; Jones et al., 2022; Li et al., 2022; Schang et al., 2021) or readily available in the laboratory facilities were employed to conduct a comparative evaluation. The materials, either synthetic or organic, exhibit a range of physical and chemical properties as described in Table 1.

Membranes were purchased in the form of disks with a surface of about 17 cm^2. Cotton gauzes and clay aggregates were normalized to cover an equivalent adsorbent surface. The materials (2 technical replicates each) were immersed in dedicated bottles containing 100 ml of contaminated freshwater under gentle movement at room temperature (24 °C ± 1). Twenty-four hours post-deployment (hpd), the materials were collected and analyzed individually in accordance with protocol #1. In detail, the materials were immersed in 2 ml NucliSENS easyMAG Lysis Buffer (bioMérieux, Marcy-l'Étoile, France) and vortexed for 1 min. They were then squeezed against the wall of the tube before being disposed of. The entire resulting volume was subjected to manual nucleic acids purification using the NucliSENS easyMAG Reagents (bioMérieux, Marcy-l'Étoile, France) and a 12-tube magnetic separation rack (New England Biolabs, Ipswich, MA, USA), according to the steps described in Farkas et al. (2021) with minor volume modifications.

A representative subset of materials showing the best performance (i.e., clay 'CLA', cotton gauzes 'CoG', nitrocellulose 'NC', nylon 'NYL' and polycarbonate 'PC') was selected to compare the efficacy of two additional laboratory procedures. In protocol #2, the materials were immersed in 15 ml buffer with glycine 0.25 M at pH 8 and glass PowerBeads 0.1 mm (Qiagen, Hilden, Germany). The samples were vortexed for 1 min and clarified at $4731 \times g$ for an additional minute. The resulting supernatant was concentrated with unirradiated pipettes with pores < 0.05 µm on a CP Select Automated System and eluted with the PBS-based FluidPrep Elution Buffer (Innovaprep, Drexel, MO, USA) to obtain approximately 300 µl of sample. Prior concentration, the pipettes were primed by soaking on a 0.1% Tween 20 solution (Sigma-Aldrich, Taufkirchen, Germany) for 5 min. The nucleic acids were isolated with the MagMAX Pathogen RNA/DNA Kit (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) (protocol 'Low-cell content sample') on a KingFisher Flex Purification System (Thermo Fisher Scientific, Waltham, MA, USA). In protocol #3, the materials were immersed in 15 ml phosphate-buffered

Table 1 List and properties of the adsorbent materials used in the study

Abbreviation	Commercial denomination	Composition/properties
CLA	Expanded clay (Cifo, San Giorgio di Piano, Italy)	Inert porous large surface area with unspecific interactions Resistance to different pH values and solvents, and to temperature variations without chemical or physical changes
CoG	Gauze compresse (PVS First Aid, Cassina de Pecchi, Italy)	Gauze with 8 plies High hydrophilicity and adsorbency
NC	Cellulose nitrate membrane filters (Sartorius, Göttingen, Germany)	Combination of cellulose nitrate and cellulose acetate High hydrophilicity and low adsorbency with effective retention of microorganisms Pore Size 0.2 µm
NYL	Nylon membrane filters (Sartorius, Göttingen, Germany)	Polyamide Hydrophilicity and very high non-specific adsorbency with chemical resistance to alkaline solutions and organic solvents Pore Size: 0.2 µm
PC	Polycarbonate Membrane Filter (Merck, Darmstadt, Germany)	Polycarbonate Hydrophilicity and non-hygroscopic Pore Size: 0.8 µm
PES	Membrane Filter Express plus (Merck, Darmstadt, Germany)	Polyethersulfone Hydrophilicity with low protein-binding capacity Pore Size: 0.2 µm
RNC	Whatman regenerated cellulose membrane (Cytiva Life Sciences, Marlborough, MA, USA)	Cellulose acetate Spontaneous wetting and wide compatibility with organic and aqueous media Pore Size: 0.2 µm
SP	Natural marine sponge	Organic porous surface High hydrophilicity
ZP	Zeta PlusVR Series (3 M, St. Paul, MN, USA)	Inorganic particles, cellulose, and crosslinking polymer Positively charged Adsorbency

For each material, the relevant physical and chemical properties as declared by the manufacturer are reported

saline (PBS) at pH 7.2 with 0.1% Tween 20 (Sigma-Aldrich, Taufkirchen, Germany) and 0.001% antifoam L-30 emulsion (Sigma-Aldrich, Taufkirchen, Germany), and vortexed for 1 min. The materials were then removed and the remaining suspension was concentrated using primed and unirradiated ultrafiltrating pipettes and subjected to nucleic acids purification as described in protocol #2.

In all the laboratory protocols, the intype IC-RNA internal control (Indical Bioscience, Leipzig, Germany) was added to the lysate in a 1:10 v/v ratio with respect to the elution volume, and the purified nucleic acids were treated with the One-Step PCR Inhibitor Removal Kit (Zymo Research, Tustin, CA, USA). A qRT-PCR targeting the AIV M gene (Heine et al., 2015) was used to infer viral load. To ensure the reliability of nucleic acid extraction and amplification and to monitor the detrimental impact of PCR inhibitors, the AIV M gene was co-amplified with the intype IC-RNA internal control as detailed in Laconi et al. (2020). qRT-PCRs were performed in duplicate obtaining four observations per testing condition. All qRT-PCR runs were performed on a CFX96 Touch Deep Well Real-time PCR Detection System and analyzed with the Bio-Rad CFX Manager software (Version 3.1) (Bio-Rad Laboratories, Hercules, CA, USA). The best laboratory procedure (protocol #1, see Sect. "Qualitative screening of the different adsorbent materials") was used for all subsequent experiments.

Determination of the Limit of Detection of Passive Samplers

Based on preliminary results, cotton gauzes were employed as a prototype material to determine their analytical sensitivity as PS. Three gauzes (17 cm^2 adsorbent surface area each) were immersed in individual glass bottles containing 100 ml brackish water spiked with decreasing amounts of H10N1 within the range of 10^7 and 10^3 GC/L, and incubated for 24 h at room temperature (24 °C ± 1) under gentle shaking. Subsequently, gauzes were eluted individually with protocol #1 and subjected to qRT-PCR in multiple replicates.

Comparison of Different Materials and Deployment Times Using Torpedo Devices

The five materials that had exhibited the highest virus recovery during the preliminary experiments were tested under different deployment times to establish their optimal

conditions of use as PS. Experiments were carried out at the fish facilities of the IZSVe. Polyurethane tanks arranged in a G-Hub System were filled with 2.5L of H10N1-contaminated water ($n=4$ freshwater tanks and $n=4$ brackish water tanks) and individually connected to recirculation pumps without filters to create water movement. To obtain technical replicates, each material was embedded into two different polyethylene terephthalate torpedo devices (Monash University, Clayton, Australia), resulting in a total adsorbent surface area of 34 cm^2 each. Both torpedoes were then immersed in water tanks for periods of either 3 h, 24 h, or 7 days at room temperature (21 °C ± 1). Fig. S1 provides an overview of the experimental set up.

To assess any loss of captured virus from PS in a simulated dynamic ecosystem, an additional experiment was set up by deploying the torpedoes for a period of 24 h into AIV-contaminated water followed by transfer into clean water tanks for additional 24 h. Data obtained from the materials deployed for 24 h in contaminated water without any transfer onto clean tanks were used as a comparison to infer virus loss.

At the designated time points, torpedoes were gathered from tanks. Additionally, 500 µl of water was collected at the beginning of the experiment (T0) and at each time point from all the tanks to measure viral decay over time (referred as 'control water' CW). The adsorbent materials were processed individually with protocol #1. Water samples were subjected to nucleic acids purification with the NucliSENS easyMAG Reagents (bioMérieux, Marcy-l'Étoile, France). All specimens were tested by qRT-PCR in duplicate.

Evaluation of the Capacity of Passive Samplers to Adsorb Degraded AIV

Five hundred µl aliquots of a H10N1 virus batch (initial titer: 8.3×10^6 *foci* forming units, FFU) were subjected to incubation at a temperature of 56 °C for 5 min, 10 min, 15 min, 20 min, 25 min, and 30 min. In order to assess the structural degradation of viral particles after the heat treatment, transmission electron microscopy (TEM) was employed using a Philips EM 208 instrument (Philips, Amsterdam, the Netherlands). Briefly, 100 µl of viral suspension were centrifuged for 2 min with a 200-mesh copper grid with formvar and carbon coatings (Electron Microscopy Sciences, Hatfield, PA, USA). After blotting the liquid in excess, samples were subjected to negative staining for 1 min with a 2% solution of sodium phosphotungstate that was removed immediately after. The grid was subsequently dried for 5 min and examined at 80 kV.

The loss of viral infectivity consequent to inactivation was verified by a plaque-forming unit assay in MDCK cell monolayers (Matrosovich et al., 2006). The treatment that resulted in the degradation of virions and consequent loss of infectivity without affecting genome detectability by qRT-PCR, was applied to the H10N1 batch for the adsorption experiments. Briefly, the heat-treated virus was diluted 1:100,000 v/v into a bottle containing 100 ml of brackish water. Cotton gauzes and nylon membranes ($n=3$ pieces of 17 cm^2 for each material) were immersed in the solution under slow movement at room temperature (24 °C ± 1). After the incubation period, membranes were analyzed in accordance with protocol #1 and subsequent qRT-PCR in duplicate. A control water spiked with the same batch of virus, but without prior heat treatment, was set up and processed in parallel.

Statistical Analysis

Due to the high number of combinations tested, the possibility to replicate the experiments was constrained by logistical and biosafety limitations. For this reason, data related to individual experiments were analyzed qualitatively. The mean Cq values and standard deviations (SD) were calculated using all the scores obtained from the replicate materials and qRT-PCR testing. Data were then examined in terms of shift of Cq values (delta Cq, ΔCq) with respect to a comparator condition.

With the aim of identifying the adsorbent materials with the highest performance regardless of the water type and experimental setting, we aggregated data from the trials described in Sects. "Preliminary selection of adsorbent materials and laboratory protocols" and "Comparison of different materials and deployment times using torpedo devices" related to the 24-hour deployment period (Figs. 1 and 2). The comparisons among materials were conducted using Welch's ANOVA followed by the Dunnett's T3 test. All graphs and statistical analyses were made with GraphPad Prism (Boston, MA, USA) version 10.

Use of Passive Samplers for AIV Monitoring in Wetlands

Valle Figheri (Venice) is a recognized wintering site for waterfowl which hosts a bird ringing station operating on a regular basis during the migration seasons. This site also lends itself to the collection of swabs for the AI active surveillance program in wild birds gathered in the national territory. Accordingly, this site was selected for field validation of PS by direct comparison with data obtained from birds sampling carried out from October 1st to November 19th, 2024.

The birds were captured weekly using an Abberton trap for subsequent ringing and recording of relevant ecological data by trained ornithologists and veterinarians. Additionally, tracheal, cloacal, and feather swabs were collected in

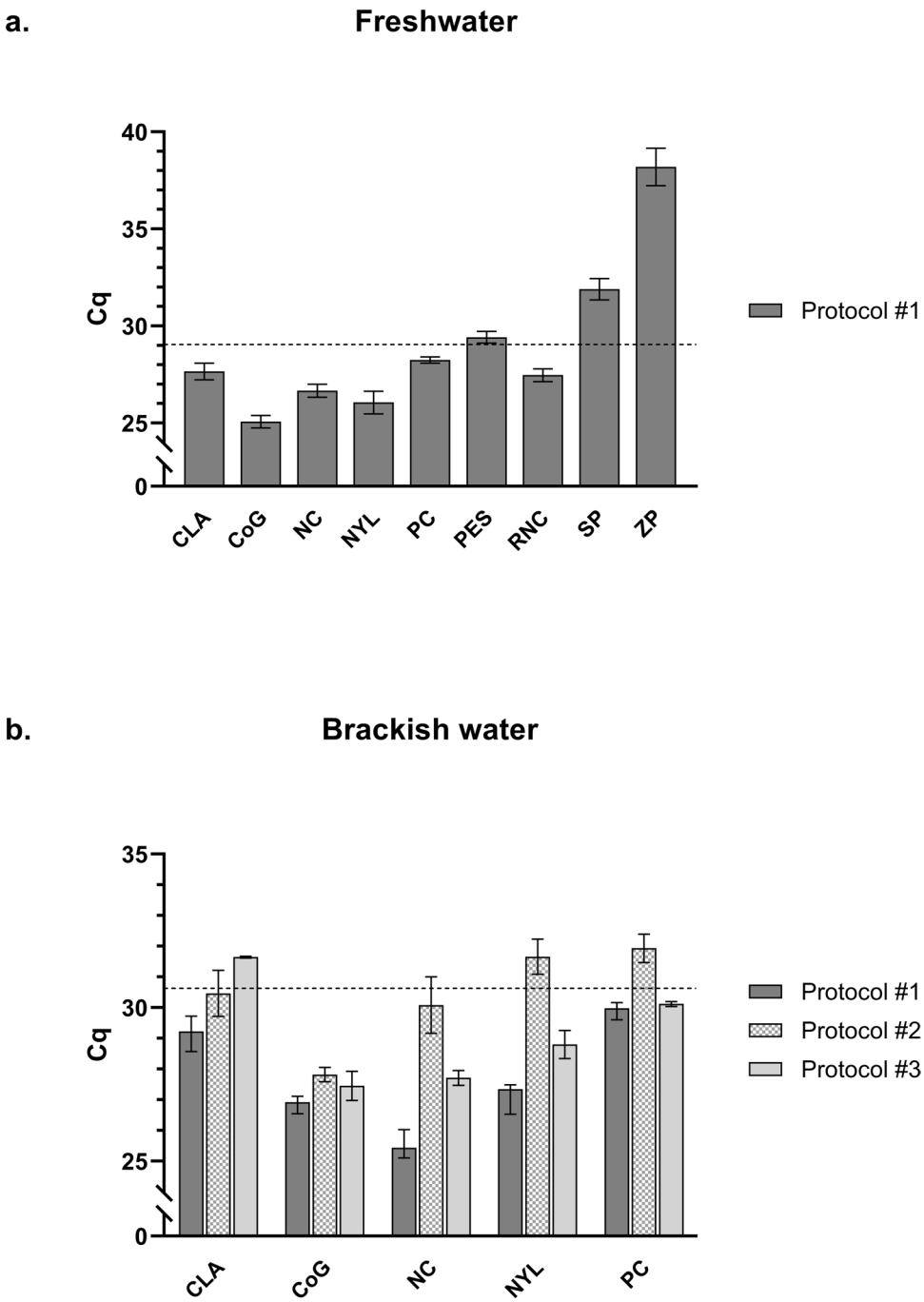

Fig. 1 Screening of different adsorbent materials. **a** Evaluation of the adsorbent capacity of materials deployed in AIV-contaminated freshwater for 24 h and processed with protocol #1. **b** Comparison of three different laboratory protocols employed to process materials after deployment for 24 h in AIV-contaminated brackish water. The types of materials and qRT-PCR Cq values are reported along the *x*-axis and *y*-axis, respectively. Bars represent the amount of recovered virus after the processing of deployed materials expressed as mean Cq ± SD. The dotted line corresponds to the mean Cq value measured in the spiked water at T0. *CLA* expanded clay, *CoG* cotton gauze, *NC* nitrocellulose, *NYL* nylon, *PC* polycarbonate, *PES* polyethersulfone, *RNC* regenerated cellulose acetate, *SP* natural sponge, *ZP* Zeta PlusVR

accordance with the Guidelines to the use of wild birds in research (Fair et al., 2023) to minimize distress.

For field deployment of PS, torpedoes were filled with the best adsorbent materials (i.e., cotton gauzes, nitrocellulose, nylon) that were selected based on previous laboratory validation data. Devices were then linked to polystyrene floats to enable contact between the embedded materials (51 cm^2 of equivalent adsorbent surface) and the surface water and were positioned with a lead weight in different water ponds at a distance from the trap. Torpedoes (one per pond) were installed during the weekly bird sampling activities and were deployed for either 3 h or 7 days, depending on the availability of the staff involved in the field operations. In parallel, the FluidPrep EasyElute—Large Volume Concentration (LVC) Kit (Innovaprep, Drexel, MO, USA) was connected to a 12 VDC advanced peristaltic pump (Royal Eijkelkamp, Giesbeek, Netherlands) set at 150 rpm and < 10 PSI to ultrafiltrate the water surrounding the trap at a rate of 1L/min. Parameters were set in conformity with the requirements of the LVC system. Pumping was performed for the

whole duration of the birds swabbing operations, totaling approximately 50L of filtrated water. The field elution with the PBS-based EasyElute Elution Buffer yielded 70–100 ml sample.

Upon collection, samples were sent to the laboratory at refrigerated transport temperature for immediate testing. Field-deployed PS were processed in accordance with protocol #1. Regardless of the quantification cycle obtained (Cq) with the M gene screening assay, subtype determination was performed according to Hassan et al. (2022) and Panzarin et al. (2022). RT-PCR (Slomka et al., 2007) followed by Sanger sequencing was applied to specimens yielding positive results for the H5 target to determine the virus pathotype. Ultrafiltrated water samples were further concentrated using the CP Select Automated System according to the above methodology (Sect. "Preliminary selection of adsorbent materials and laboratory protocols"). The obtained volume (400–500 µl) was then processed with the NucliSENS easyMAG Reagents (bioMérieux, Marcy-l'Étoile, France) according to protocol #1 and subsequent qRT-PCR.

Bird swabs were dipped into 750–1000 µl of PBS supplemented with antibiotics and antimycotics and swirled for a few seconds to allow virus release. For cloacal swabs visibly soiled with feces, a centrifugation step at 18,000 × *g* for 2 min was also performed. Two hundred µl were collected from the suspension and subjected to nucleic acids extraction according to the protocol employed at the IZSVe for routine diagnosis (MagMAX Pathogen RNA/DNA Kit, Applied Biosystems—protocol 'Low-cell content sample'). Birds were considered AIV-positive when yielding Cq ≤ 36 in tracheal and/or cloacal swabs with the M gene qRT-PCR. Any positive detection in feather swabs only was interpreted as an indication of environmental contamination. Regardless of the type of swab, samples yielding Cq ≤ 36 were subjected to H5 testing only according to Slomka et al. (2007).

Results

Qualitative Screening of the Different Adsorbent Materials

The array of materials deployed in freshwater and processed with protocol #1 demonstrated a wide range of recovery efficiencies, as illustrated in Fig. 1a. For most materials, the amount of eluted virus at 24 hpd and measured by qRT-PCR was higher compared to the one detectable in the spiked water at the beginning of the experiment (ΔCq_{T0}), which proves their adsorption ability as PS. In particular, cotton gauzes (CoG) showed the best recovery, recording a ΔCq_{T0} of 3.99, followed by nylon (NYL) ($\Delta Cq_{T0} = 3.00$) and nitrocellulose (NC) ($\Delta Cq_{T0} = 2.39$). In contrast, polyethersulfone (PES), sponge (SP) and Zeta Plus VR (ZP) recorded negative ΔCq_{T0} values corresponding to − 0.36, − 2.70, and − 9.13, respectively. In view of these observations, and of the higher recovery of nitrocellulose (NC) over regenerated nitrocellulose (RNC), only clay, cotton gauzes, nitrocellulose, nylon, and polycarbonate were selected as representative materials with positive ΔCq_{T0} for downstream validation trials.

To run a direct comparison of how different elution and extraction procedures might influence the recovery efficiency, the designated materials were deployed in spiked brackish water and tested in parallel with protocols #1, #2, and #3. In this experiment, we demonstrated that protocol #1, which involved direct elution with the NucliSENS easyMAG Lysis Buffer followed by nucleic acid extraction with the NucliSENS easyMAG Reagents (bioMérieux, Marcy-l'Étoile, France), consistently outperformed the other two protocols (Fig. 1b). Therefore, protocol #1 was employed to process PS throughout the entire study.

Detection Limit of Passive Samplers

The amount of virus that could be adsorbed by cotton gauzes deployed for 24 h in brackish water is presented in Table 2. Reliable recovery was obtained from gauzes immersed in water spiked with 10^7–10^5 GC/L of virus, with optimal linearity within the range ($R^2 = 0.98$). At a lower contamination level (10^4 GC/L), the spiked water was consistently negative,

Table 2 Lowest detection limit of passive samplers

	H10N1 load in the water sample (GC/L)				
	10^7	10^6	10^5	10^4	10^3
Spiked water	32.33 ± 0.03 (2/2)	35.44 ± 0.15 (2/2)	37.57 (1/2)	Neg (0/2)	Neg (0/2)
Eluted virus	27.97 ± 0.39 (6/6)	31.66 ± 0.29 (6/6)	35.02 ± 0.55 (6/6)	37.81 ± 0.76 (7/12)	38.20 (1/12)

qRT-PCR testing was performed on the spiked water at T0 and on the eluted virus. Measurements are reported as mean Cq ± SD. The number of qRT-PCR replicates yielding positive results is reported in brackets

while eluates from the cotton gauzes yielded positive results in 58.3% of the qRT-PCR replicates (7/12). Of the gauzes deployed in water contaminated with 10^3 GC/L AIV, only one qRT-PCR replicate tested positive.

Determination of the Effect of Deployment Time on Virus Adsorption

This experiment had the aim of determining under controlled conditions the efficacy of PS for different deployment times, after the occurrence of a single contamination event (Fig. 2 and S2, Table S1). To achieve this goal, we analyzed the recovery of each material with respect to the viral loads measures by qRT-PCR in the spiked water at either T0 (ΔCq_{T0}) or at each time point (ΔCq_{CW}).

In brackish water, we observed that from the initial value of 29.34 Cq at T0, at 3 hpd, 24 hpd and 7 dpd viral loads progressively decreased in the tanks, recording Cq values of 29.65, 30.23, and 35.92, respectively (Fig. 2, Table S1). When comparing the eluted virus from each material with the corresponding control water at the designated time points, the best recovery was obtained for the cotton gauzes, scoring ΔCq_{CW} values of 4.21 and 6.01, at 3 and 24 hpd, respectively. After 7 days from spiking, the best performing materials were cotton gauzes, nitrocellulose, and nylon, which recorded similar ΔCq_{CW} values ranging from 5.64 to 6.23. In freshwater, we observed that from the initial load of 29.35 Cq at T0, at 3 hpd and 24 hpd viral loads decreased to 29.59 and 30.24 values, respectively. After 7 days, no trace of viral genome was detected in the water. The comparison of materials showed that after 3 h all materials, except for clay, performed similarly, recording a ΔCq_{CW} ranging 2.61–3.30. At a 24-hour interval, the cotton gauzes recorded the highest recovery, with a ΔCq_{CW} of 5.04, followed by nylon ($\Delta Cq_{CW} = 3.37$). After 7 days, the highest recovery was observed for cotton gauzes, nylon and nitrocellulose, scoring similar Cq

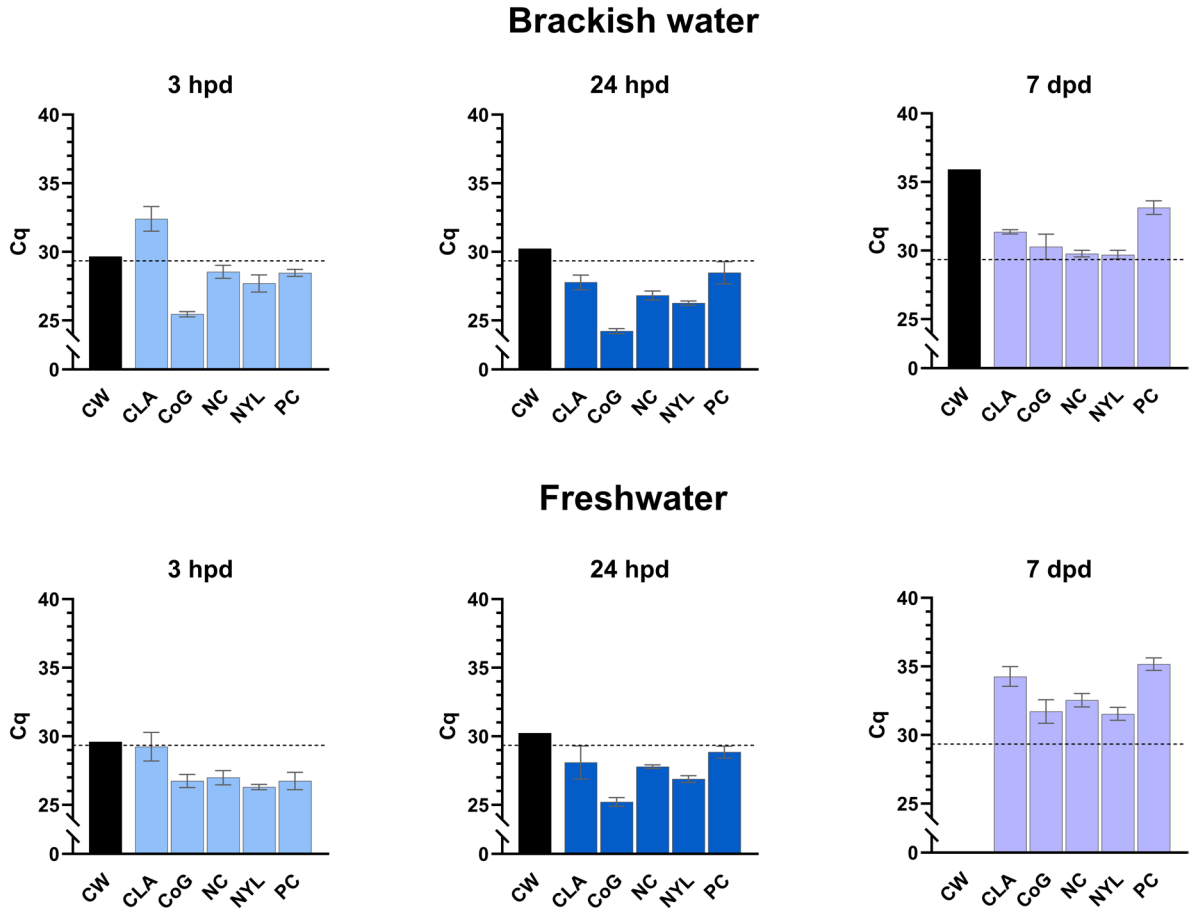

Fig. 2 Adsorbent capacity of PS upon a single contamination event. For both brackish water and freshwater, data are presented to compare the performance of the different materials at each deployment time. The designated materials and qRT-PCR Cq values are reported along the *x*-axis and *y*-axis, respectively. Colored bars represent the mean Cq ± SD of the virus eluted from PS. The black bars refer to the viral load in the control water (CW) at each time point. The dotted line corresponds to the mean Cq value measured in the spiked water at T0. *CLA* expanded clay; *CoG* cotton gauze, *NC* nitrocellulose, *NYL* nylon, *PC* polycarbonate

values ranging 31.54–32.54, and for which no ΔCq_{CW} was calculated as the control water had been found negative.

When analyzing more in detail the effect of time on the performance of each single material (Fig. S2, Table S1), we observed that, except for clay, all materials effectively adsorbed the virus in brackish water improving its detection already at 3 hpd, with ΔCq_{T0} values ranging 0.80–3.90. For cotton gauze, nitrocellulose and nylon, the recovery reached the highest level at 24 h from deployment, with ΔCq_{T0} values ranging 2.53–5.12, while 7 days after spiking, the same materials recorded Cq values similar to that detected in spiked water at T0 (ΔCq_{T0} between -0.35 and -0.94). For polycarbonate and clay, after 7 days, the Cq were 3.79 and 2.03 cycles higher than the initial value of spiked water at T0, respectively. Overall, the greatest positive ΔCq_{T0} of 5.12 was observed with the cotton gauzes, after 24 h from deployment. In freshwater, we observed that after 3 h from deployment, all materials, except for clay, recorded lower Cq values than that obtained from the spiked water at T0. ΔCq_{T0} were similar and ranged between 2.37 and 3.06. Of these materials, only the cotton gauzes further increased their recovery at 24 hpd, recording a ΔCq_{T0} of 4.15, which represented the greatest positive ΔCq_{T0} in freshwater. After 7 days, all materials showed Cq values that were at least 2.19 cycles higher than the one initially detected in spiked water at T0.

In order to identify the materials yielding the highest recovery on a broader level, the data recorded for laboratory-batch experiments based on a 24-h deployment period (Sect. "Preliminary selection of adsorbent materials and laboratory protocols") were aggregated with those obtained from the deployment of the materials in 2.5L spiked water for the same duration (Fig. 3). Of note, cotton gauzes, nitrocellulose and nylon yielded greater virus recovery with Cq values ranging between 25.33 and 26.68. No statistically relevant differences in the recovery were observed between these materials, although cotton gauzes yielded the lowest mean Cq value (25.33 ± 1.04). In contrast, clay and polycarbonate recorded significantly higher Cq values (28.17 ± 0.95 and 28.86 ± 0.80, respectively) ($p \leq 0.001$).

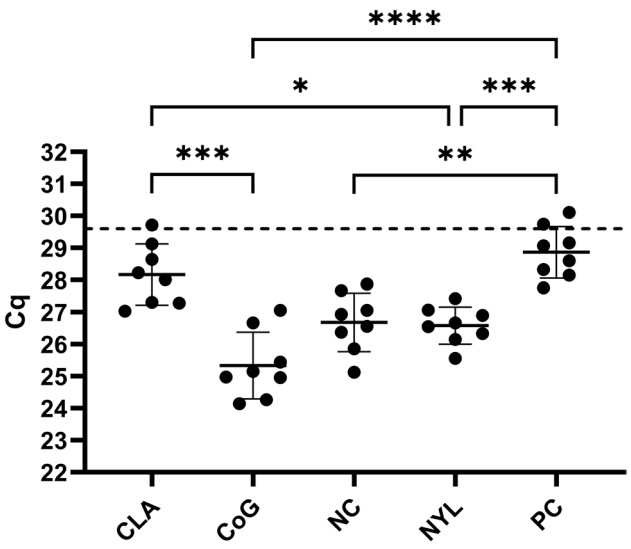

Fig. 3 Overall comparison of the performance of adsorbent materials at 24 hpd. Data from Sects. "Preliminary selection of adsorbent materials and laboratory protocols" and "Comparison of different materials and deployment times using torpedo devices" related to a period of deployment of 24 h were aggregated regardless of the water type and the experimental settings. Dots represent individual Cq values of eluted virus, and horizontal bars report the mean Cq ± SD. The dotted line corresponds to the mean Cq of the spiked water at T0 related to all the experiments. Significant differences in multiple comparisons are marked with asterisks (* = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$). *CLA* expanded clay, *CoG* cotton gauze, *NC* nitrocellulose, *NYL* nylon, *PC* polycarbonate

Estimation of Net Virus Loss During Deployment in Clean Water

Torpedoes that had been deployed for 24 h in spiked water were subsequently transferred to tanks containing an identical volume of clean water. After another 24 h, PS were collected and processed for elution and qRT-PCR to infer virus loss consequent to virus washout after water change (Fig. 4). In the case of brackish waters, all the materials had varying degrees of virus loss. Clay, cotton gauzes, nitrocellulose, nylon and polycarbonate had Cq values that differed from the values recorded at 24 hpd by 1.84, 4.35, 3.63, 2.84 and 1.55 cycles. Interestingly, cotton gauzes and nylon were the materials with the highest recovery despite their noticeable loss, recording Cq values of 28.15 and 27.73, respectively, after their transfer into clean water. The same experiment conducted in freshwater indicated that clay, cotton gauzes, nitrocellulose, nylon and polycarbonate differed from the 24 hpd comparator by 1.39, 2.94, 1.37, 0.86 and 2.45 cycles. Also in this case, cotton gauzes and nylon were the two materials yielding the highest recovery after washout by tank change (Cq = 28.56 and 29.09, respectively).

Impact of AIV Integrity on the Adsorbent Capacity of PS

To assess whether the structural integrity of AIV might impair the ability of materials to perform as PS, we deployed two of the best performing substrates in brackish water spiked with either intact H10N1 virus or heat-treated H10N1 virus. Figure 5a presents TEM micrographs and titration in MDCK monolayers related to both untreated and heat-treated H10N1 batches. Incubation of the virus at 56 °C for 30 min resulted in the disruption of the envelope and in the presence of proteins amorphous aggregates. The absence of *foci*, as evidenced by MDCK staining, indicated that infectivity had been abolished without significant degradation of

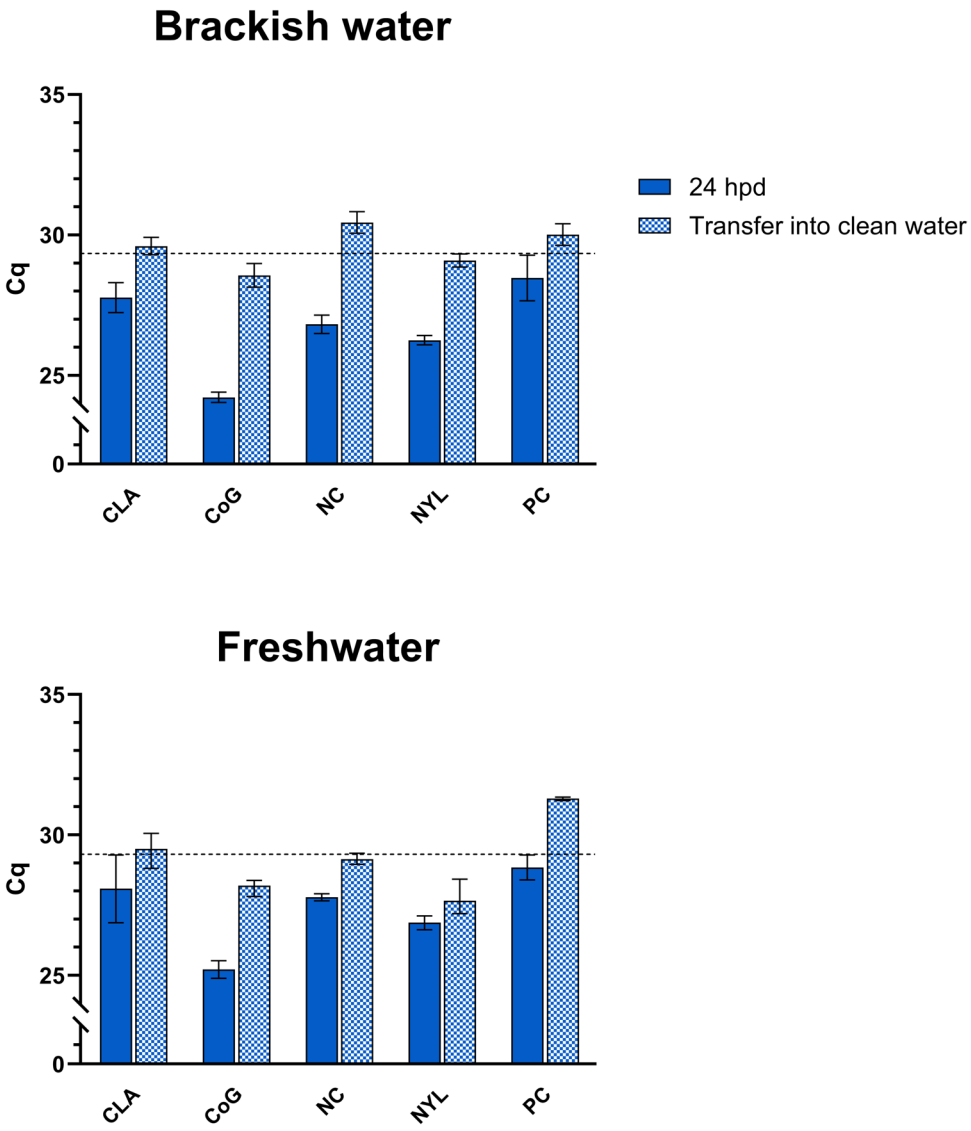

Fig. 4 Effect of washout on virus recovery from adsorbent materials. For both brackish water and freshwater, data are presented to show the loss of adsorbed virus by effect of complete water change (light bars), compared to the virus recovery after 24 hpd without any system perturbation (dark bars). The designated materials and qRT-PCR Cq values are reported along the *x*-axis and *y*-axis, respectively. Bars represent the mean Cq ± SD of the eluted virus from PS at the different treatment conditions. The dotted line corresponds to the mean Cq value measured in the spiked water at T0. *CLA* expanded clay, *CoG* cotton gauze, *NC* nitrocellulose, *NYL* nylon, *PC* polycarbonate

the virus genome (untreated H10N1: 12.16 ± 0.05 Cq; heat-treated H10N1: 12.42 ± 0.11 Cq). qRT-PCR testing showed that cotton gauzes and nylon were effective in adsorbing both structurally intact and degraded viruses, with no relevant loss of recovery (Fig. 5b). In particular, cotton gauzes and nylon recorded ΔCq between the two experimental conditions of 0.28 and 0.41 cycles, respectively.

Field Testing of Passive Samplers

Environmental and animal testing data are presented in Tables 3 and Table S2. Pictures of torpedoes assembly and deployment, and on-site water ultrafiltration are provided in Fig. 6.

Overall, AIV genome was detected in 16 out of 30 PS (53.3%) from all ponds, with Cq ranging between 33.88 and 39.18. Downstream qRT-PCR characterization allowed the identification of the H5/H12/N2/N5/N6/N9 subtypes.

On October 1st 2024, a torpedo fitted with cotton gauze and nylon was deployed for a period of 3 h in a pond where the trap for capturing birds was positioned. AIV RNA (N6 subtype) was only detectable in the cotton gauze. On the same day, 2 Eurasian teal individuals yielding 28.4–32.14 Cq in tracheal and/or cloacal swabs tested positive for H5 HPAI. In the period 8th–29th October, the weekly testing of PS revealed the presence of AIV genome in 8 out of 16 specimens (H5/N9 subtypes), although the small number of captured animals prevented any comparison with traditional swab testing. In November 2024, AIV RNA was detected in 7 out of 12 PS. For 2 specimens testing positive for the H5 target by qRT-PCR, the HA cleavage site (CS) sequence was obtained (PLREKRRKR/GLF), confirming the presence of HPAI molecular motifs. Of the 34 matching birds captured during the same period, 2

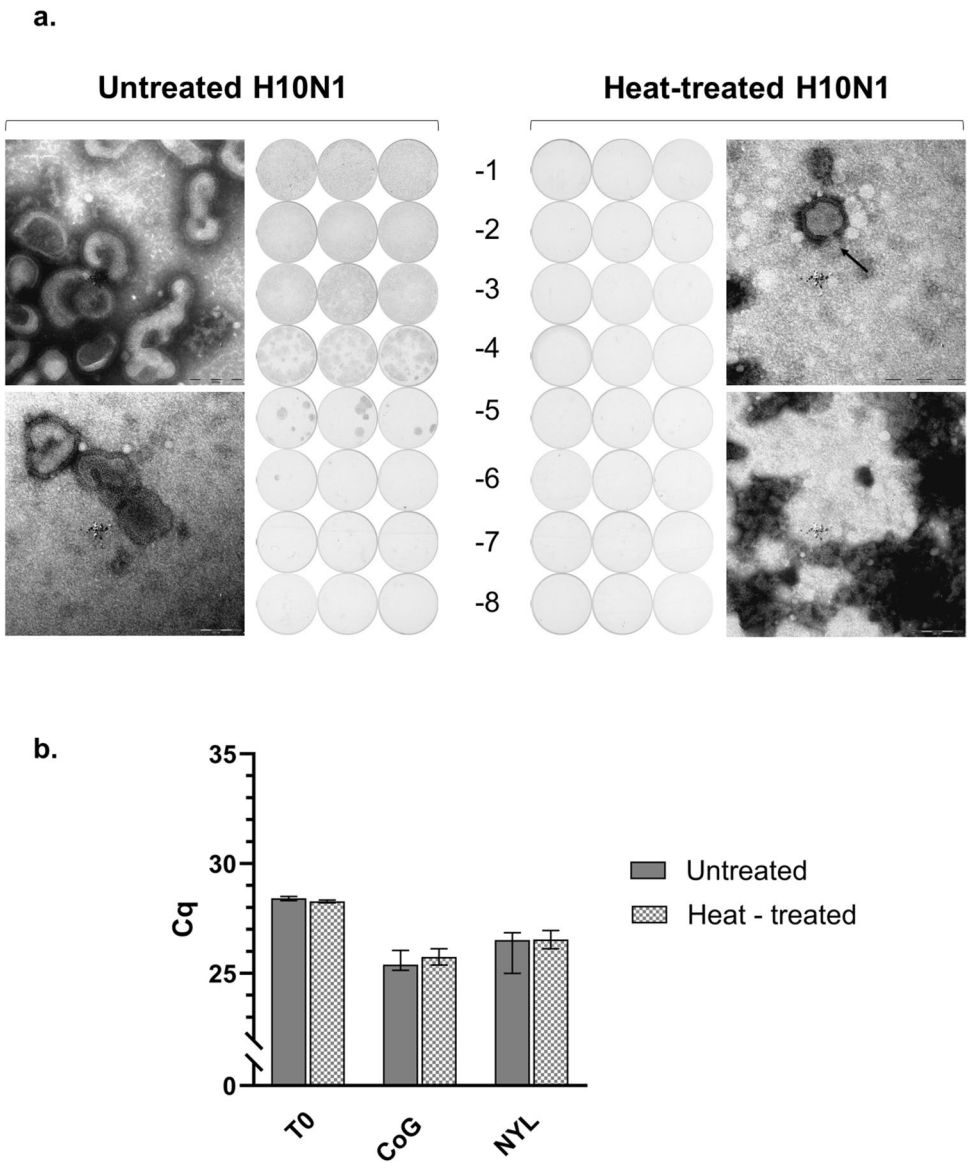

Fig. 5 Evaluation of the capacity of adsorbent materials to capture degraded AIV. **a** TEM micrographs (71 or 89 kx magnification) and MDCK titration of untreated and heat-treated H10N1 (56 °C for 30 min). Numbers refer to Log10 dilutions of the viral suspension. The arrow indicates the damaged virus envelope. Images related to intermediate incubation times are not shown. **b** Deployment of cotton gauzes (*CoG*) and nylon (*NYL*) membranes in brackish water contaminated either with intact (untreated) and heat-treated virus. Bars represent the mean Cq ± SD measured in the spiked water at T0 and the eluted virus from the different adsorbent materials (*x*-axis)

tested positive for AIV in tracheal and/or cloacal swabs. The individual identified by the ring number TP4613 was found to be positive for H5 HPAI. Interestingly, AIV RNA signal was identified in the plumage of 5 birds with Cq within the range 33.75–36.58, and in 2 cases, it was possible to obtain a HA CS sequence identical to the one of PS. It is noteworthy that none of the water samples ultrafiltrated on-site and subsequently concentrated in the laboratory allowed the detection of AIV genome in the environment.

Discussion

Surveillance in wild birds is of paramount importance for the early detection of highly pathogenic avian influenza (HPAI) and for the implementation of risk-based biosecurity measures in poultry holdings (EFSA, 2024b). Nevertheless, the monitoring of wild avifauna is predominantly limited to the opportunistic testing of carcasses, which is not sufficient for a comprehensive understanding of the prevalence of HPAI (Kuiken, 2024). The complementary testing of captured and hunter-harvested wild birds can significantly enhance the coverage of surveillance, providing substantial insights into disease ecology and epidemiology (Giacinti et al., 2024; Trogu et al., 2024); however, it should be noted that such campaigns rely on the voluntary efforts of individual countries, which require adequate economic support, infrastructures, and the collaboration of professional ornithologists and hunter associations for their successful implementation. Another option to improve the breadth and timeliness of surveillance for HPAI is to conduct environmental

Table 3 Deployment of passive samplers a wetland

Date	Adsorbent material	Deployment time	Pond	Cq	Subtype	Ultrafiltrated water	AIV-positive birds
01/10/24	Cotton gauze	3 h	A	37.17	N6	Neg	2/21
	Nylon	3 h	A	Neg	n.p		
08/10/24	Cotton gauze	7 days	B	39.17	H5	Neg	0/2
	Nylon	7 days	B	Neg	–		
	Cotton gauze	7 days	C	Neg	–		
	Nylon	7 days	C	37.47	n.t		
15/10/24	Nylon	7 days	A	38.18	n.t	Neg	0/1
	Cotton gauze	7 days	A	37.78	n.t		
	Nylon	7 days	B	36.14	N9		
	Cotton gauze	7 days	B	38.51	n.t		
	Nylon	7 days	C	Neg	–		
	Cotton gauze	7 days	C	39.18	n.t		
22/10/24	Nylon	7 days	B	Neg	–	Not performed	0/2
	Nitrocellulose	7 days	B	Neg	–		
	Nylon	7 days	C	Neg	–		
	Nitrocellulose	7 days	C	Neg	–		
29/10/24	Nylon	7 days	C	Neg	–	Neg	0/0
	Nitrocellulose	7 days	C	37.56	n.t		
05/11/24	Nylon	7 days	B	Neg	–	Neg	0/9
	Nitrocellulose	7 days	B	Neg	–		
	Nylon	7 days	C	Neg	–		
	Nitrocellulose	7 days	C	38.1	n.t		
12/11/24	Nylon	7 days	A	34.68	H5[a]	Neg	2/9
	Nylon	7 days	A	33.88	H5[a]/N2/N5		
	Nylon	7 days	C	Neg	–		
	Nylon	7 days	C	Neg	–		
19/11/24	Nylon	7 days	A	34.79	H12/N5	Neg	0/16
	Nylon	7 days	A	35.22	N5		
	Nylon	7 days	B	38.21	H5		
	Nylon	7 days	B	36.84	H5		

[a]H5 HPAI genome confirmed by RT-PCR followed by Sanger sequencing

For each sampling event, the date of collection in different ponds, the deployment time and the presence of AIV-positive wild birds are indicated. The amount of virus eluted from PS is reported as Cq. *n.p.* subtyping not performed, *n.t.* not typable. A = pond where the Abberton trap is also located; B = pond at a distance of 70 mt east from A; C = pond at a distance of 500 mt north from A

monitoring as a supplementary strategy to traditional animal testing (Duan et al., 2023). Target substrates for laboratory testing include surface water, which has been recognized as conducive to the maintenance and transmission of AIV in waterfowls (Ahrens et al., 2022; Kenmoe et al., 2024). In this context, we have introduced the use of PS as a cost-effective and user-friendly tool for the detection of AIV in water bodies, where the application of traditional concentration methods may face logistical challenges or do not significantly improve virus detection and typing (McCuen et al., 2021).

Cotton Gauzes, Nitrocellulose, and Nylon Are Effective as PS and Can Accommodate Different Deployment Periods

The results of our laboratory experiments demonstrated that the materials tested have different efficiency in capturing and recovering AIV. In particular, our initial screening after 24-hour deployment in freshwater indicated that natural marine sponge and a commercial filter made of cellulose combined with a positively charged crosslinker

Fig. 6 Water field sampling. **a** Torpedoes were filled with cotton gauzes (up) or nylon/nitrocellulose disks (bottom). The lid was equipped with a floating material to prevent the device from sinking. **b** Torpedoes were attached to polystyrene elements to float on the surface of the water and were positioned on the pond with a lead weight for 3 h or 7 days. **c** Torpedo retrieved after a deployment period of 7 days in a wetland. **d** Field installation for on-site water ultrafiltration

polymer were poorly effective as sampling materials. So far, sponge has been scarcely investigated as PS and its inadequacy could be attributed to its propensity to deterioration, as observed in our experiments; ZetaPlus products were reported to be unsuccessful in a previous study, most likely because of the poor desorbtion that impaired the detection of enveloped viruses (Jones et al., 2022). Our initial screening also revealed that clay, cotton gauzes, nitrocellulose, nylon and polycarbonate had a remarkable ability to adsorb AIV in a contaminated water environment, and these materials were thus selected for subsequent characterization as PS under different usage conditions. In our experiment, which investigated torpedoes deployed for varying intervals in brackish or freshwater, a single spiking intervention in a closed-circuit water tank simulated a transient contamination event of avian influenza virus in a pond with limited water recycling. The aim was to establish the best deployment period of PS, and to measure the recovery achieved through this sampling strategy. Our data suggest that the deployment of cotton gauzes, nitrocellulose, or nylon in brackish water for 3 to 24 h may enhance detection in a real use scenario, compared to direct water testing at the time of contamination. Notably, after 24 h, their recovery was maximized

outperforming clay and polycarbonate irrespective of the type of water, as indicated also by the statistical analysis of aggregated data. Interestingly, previous studies on PPMoV, enterovirus, HAdV 40/14, and SARS-CoV-2 reported that virus uptake can be time-dependent, and that virus accumulation in electronegative membranes reaches the highest level after 24 to 48 h (Emalie K. Hayes et al., 2022; Li et al., 2022). Although in our experiments we did not determine the recovery of membranes and gauzes at intermediate time points, a longer deployment of 7 days resulted in a reduced yield. Nonetheless, the three materials recorded Cq values that were less than 1 cycle higher than the original contaminated brackish water at T0. This finding suggests that, in principle, the use of PS may be beneficial also upon longer deployment periods, as a similar sensitivity to direct water testing at the time of virus deposition is anticipated. Conversely, in freshwater environments, the recovery of the three materials was limited to short deployment times. In fact, after 7 days, the Cq values of the eluted virus were higher than that recorded in the water at T0 by 2.19–3.19 cycles. While our experiment aimed at determining PS efficacy after a single contamination event, it is crucial to note that the reduced recovery of AIV observed at 7 dpd represents a significant concern only in theory, if we assume that in a dynamic ecosystem, virus deposition by infected birds may occur at any time. Importantly, extended exposure of the samplers to the contaminated environment without compromising the overall sensitivity was successfully demonstrated when monitoring SARS-CoV-2 in wastewater treatment plants, where samplers deployed for up to 6 days exhibited the same efficacy as auto-composite sampling (Wilson et al., 2022).

PS Allow Sensitive AIV Detection in Water and Their Efficacy is Not Impaired By Virions Integrity

The evaluation of PS recovery for different deployment periods enabled us to collect data also on the degradation of the viral genome in contaminated water and on the impact this has on the functionality of PS. In this case, we observed that all materials, except clay at 3 hpd, had the ability to capture the virus leading to better and more sensitive detection than direct water testing at the same time point. Interestingly, after 7 days, when viral genome in brackish and freshwaters was either detected with a Cq of 35.92 or not detected at all, PS allowed to confirm AIV presence at Cq values that were at least 2.79 cycles lower. These results, are consistent with the LoD determination performed in this study and reported elsewhere (Farkas et al., 2024; Geissler et al., 2024; E. K. Hayes et al., 2021; Li et al., 2022; Mejías-Molina et al., 2023; Schang et al., 2021) and becomes of particular interest as extremely low concentrations are representative of realistic scenarios of AIV contamination. Importantly, we did not verify the integrity of virions in the control water at 7 dpd, although the qRT-PCR results obtained were strongly indicative of virus degradation. The enhanced virus detection observed for all materials, thus suggest that PS have the ability to adsorb deteriorated virions, and our subsequent trial designed to assess the effect of AIV integrity on materials absorbency, supports this assumption. However, we should stress that our experimental settings had several limitations. In fact, in our model we did not take into account the effect of UV irradiation and different temperature conditions on the degradation of viral genome. Moreover, we do not know whether or not the water contained particulates, biofilms, algae or other microorganisms that are believed to interfere with the PS ability to recover virus. In this perspective, further research is required to understand the kinetics of virus capture/release in a dynamic environment that takes into consideration virus degradation (Karamati N. et al., 2024), as well as to sound the possibility to isolate avian influenza viruses from PS for more in depth investigations. Our observations are also limited by the fact that our experiments were conducted with only one LPAI strain. For this reason, we are cautious in translating our results to infer performance of PS on other strains and subtypes of AIV, including HPAI viruses of the H5Nx subtype.

Torpedoes PS Proved Successful in Detecting AIV and H5 HPAIV Genome in Wetlands

Despite the study limitations, the laboratory-batch experiments presented in this study clearly indicated that PS efficacy may vary depending on the material, the environment and the deployment period. Consequently, end-users can tailor torpedoes embedding with single or multiple adsorbent substrates as well as the sampling scheme to better accommodate their surveillance regimen. In our field study, it was imperative to optimize efforts and resources and align PS deployment with the weekly active surveillance on captured wild birds. The test period for the evaluation of the functionality of PS under field conditions was concomitant to the beginning of the wintering season of migratory birds in the study area. Our data support the utility of PS, as torpedoes installed in wetlands allowed the successful detection of AIV genome after 3-hour or 7-day deployment even in instances where the corresponding bird swabs were negative or could not be sampled. Notably, the observed Cq values were comparable to the ones previously obtained by actively filtering 10L of surface water (Ahrens et al., 2023), confirming that PS represent an effective solution for AIV detection in water bodies without the need for pumping or sampling instrumentation. Contrarily, the ultrafiltration system used in

our field study on a larger water sample repeatedly failed to detect AIV contamination. We speculate that this outcome is primarily due to the suboptimal elution of the virus from the hollow fibers of the column, rather than to the downstream laboratory concentration step with 0.05 µm ultrafiltrating pipettes, which has previously proved effective in enriching AIV from small water samples (50–100 ml) (authors' personal data). Another relevant consideration is the high cost of these in-series ultrafiltrating systems (approximately 150€/sample, excluding the necessary equipment). The relatively low cost of a torpedo, ranging from 25 to 35€, based on its embedded material, makes it feasible to deploy more than one device in a single site or multiple locations to enhance sensitivity.

Although AIV characterization was severely hampered by the low viral load detected with PS, in a few cases it was possible to obtain partial information on the subtype composition of the samples. These include the detection of an H5 RNA in a PS installed on October 1st 2024 and collected the following week, as well as in torpedoes deployed in mid-November 2024, which confirmed the presence of the genome of H5 HPAI viruses in the environment. Importantly, during the same periods, outbreaks of H5 HPAI were reported in turkeys and in backyard flocks in the province of Venice (EURL Avian Flu Data Portal 2024), highlighting the potential of PS for early warning in avian influenza management. Substantial consistency was also observed between PS and the corresponding bird swabs collected during animal sampling activities. H5 HPAIV RNA detections were also observed in feather swabs, associated with negative tracheal and/or cloacal swabs. These data most likely reflect plumage contamination through the environment and are consistent with the viral load detected in the PS. Despite such encouraging observations, the paucity of captured birds due to the hunting season constrained the direct comparison of PS to traditional swab sampling and testing, thus preventing us from determining their relative sensitivity and specificity. It is also noteworthy that such a comparison was beyond the scope of the study, as the suggested use of PS envisages their integration within existing surveillance plans rather than the replacement of wild birds' active surveillance. However, due to their ease of use, scalability and minimal disturbance to wild habitats, PS are well-suited for surveillance in contexts where bird swabs cannot be collected, as well as for monitoring animal sanctuaries and protected areas.

One of the relevant features of PS we evaluated in this study is their capacity to retain the virus upon complete environmental water changes, similar to the ones resulting from heavy rains and tides. As expected, the exposure of PS to clean water led to a significant virus loss, particularly in brackish water, although viral particles could still be eluted from membranes allowing AIV detection. While the experimental conditions set were extreme, the observed viral retention suggests that PS have the ability to capture AIV in dynamic ecosystems where water levels may frequently change. Our field tests supported this assumption, as rains during the month of October 2024 were reported to be the heaviest of the past 30 years (ARPAV 2024). Consequently, PS are suitable for long-term *in situ* deployment where exposure to virus deposition can be either far or recent. Actually, PS also have the potential to adsorb residual environmental viruses, as demonstrated by our laboratory-batch experiments. Physicochemical and biological factors such as salinity, temperature, pH, UV, microbial activity and the viral subtype/strain are known to influence the survivability of avian influenza viruses (Dalziel et al., 2016; Warren et al., 2024). Even if the ability of PS to capture degraded viruses extends the effective timeframe for detecting AIV in the environment, inferring the recency of virus introduction remains a major limitation. A thorough temporal sampling strategy, with a larger number of torpedoes retrieved at shorter time intervals, could compensate this limitation and help to build a temporal profile of AIV presence in wild bird habitats to detect changes in trends.

Additional Prospects for Use of PS

While our field work specifically focused on PS applied to AI surveillance in wetlands taking advantage of animal samples collected in the same area to validate our data, it must be noted that their versatility permits a broader use in a variety of contexts, including rural ponds frequented by backyard/free range flocks, livestock waterers, slaughterhouses wastewater, ponds and lakes in urban parks and municipal effluents. For instance, the spread of H5 HPAI viruses of clade 2.3.4.4b in dairy cattle in the USA has prompted the monitoring of urban wastewater to assess any relevant changes in virus incidence and geographical distribution. Although H5-positive detections from composite samples were most likely due to effluents of animal origin from milk processing plants (Louis et al., 2024), the regular monitoring of municipal sewage can complement seasonal influenza surveillance and improve preparedness for the next respiratory illness seasons (Honein et al., 2024). In this regard, stakeholders and testing laboratories should consider the use of PS in place of composite sampling, as they offer a rapid and scalable solution to detect clade 2.3.4.4b viruses and have proved efficacy in a broad range of applications (Jones et al., 2022; Mejías-Molina et al., 2023; Shakallis et al., 2022).

Final Remarks

In conclusion, the laboratory-batch experiments and the pilot field evaluation herein presented indicate that PS can be an effective method for detecting avian influenza virus in water environments. The methodological approach is simple,

versatile and cost-effective, making it a valuable complement to active surveillance efforts for avian influenza in wild birds. By overcoming the limitations posed by virus dilution, complex sample transportation, and field equipment demands, PS can play an important role in environmental monitoring, especially in scenarios where capturing animals is logistically challenging. However, a field validation on a larger spatio-temporal scale is necessary to establish how environmental monitoring can be integrated into existing surveillance strategies to maximize benefits. Such verification should include testing against bird swabs as well as alternative environmental samples, such as feces, across a range of wetland types and climates, as this would strongly confirm the possibility of using PS for monitoring AIVs and eventually protect poultry production, the biodiversity, and public health.

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Author Contributions Conceptualization: VP and CT. Data curation: MC, FB, SM, AB, DF, LM, and PM. Formal analysis: MC, FB, and EM. Funding acquisition: VP, DF, PM, and CT. Investigation: VP, MC, SM, and PB. Methodology: VP, FB, SBM, and MR. Project administration: VP, FB, DF, PM, and CT. Resources: VP, FB, AB, DF, LM, PM, and CT. Supervision: VP and MR. Validation: MC, FB, SM, and EM. Visualization: VP, MC, PB, and EM. Writing—original draft: VP, MC, FB, and EM. Writing – review and editing: all the authors.

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Data Availability No datasets were generated or analyzed during the current study. All the data presented in this study are reported in the main text, tables and figures, and in the supplementary material.

Declarations

Conflict of Interest The authors declare no competing interests.

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