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Persistence of low pathogenic avian influenza virus in artificial streams mimicking natural conditions of waterfowl habitats in the Mediterranean climate

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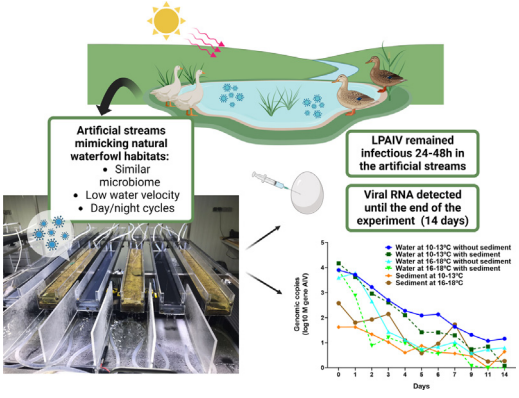
HIGHLIGHTS

- We used artificial streams to assess avian influenza environmental persistence.
- Artificial streams remained infectious between one and two days.
- Avian influenza viral RNA was detected until the end of the experiment (14 days).
- Experiments simulating real environments provide a more realistic approach.
- Mediterranean wetlands conditions could facilitate transmission during short periods.

GRAPHICAL ABSTRACT



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ABSTRACT

Avian influenza viruses (AIVs) can affect wildlife, poultry, and humans, so a One Health perspective is needed to optimize mitigation strategies. Migratory waterfowl globally spread AIVs over long distances. Therefore, the study of AIV persistence in waterfowl staging and breeding areas is key to understanding their transmission dynamics and optimizing management strategies. Here, we used artificial streams mimicking natural conditions of waterfowl habitats in the Mediterranean climate (day/night cycles of photosynthetic active radiation and temperature, low water velocity, and similar microbiome to lowland rivers and stagnant water bodies) and then manipulated temperature and sediment presence (i.e., 10–13 °C vs. 16–18 °C, and presence vs. absence of sediments). An H1N1 low pathogenic AIV (LPAIV) strain was spiked in the streams, and water and sediment samples were collected at different time points until 14 days post-spike to quantify viral RNA and detect infectious particles. Viral RNA was detected until the end

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of the experiment in both water and sediment samples. In water samples, we observed a significant combined effect of temperature and sediments in viral decay, with higher viral genome loads in colder streams without sediments. In sediment samples, we didn't observe any significant effect of temperature. In contrast to prior laboratory-controlled studies that detect longer persistence times, infectious H1N1 LPAIV was isolated in water samples till 2 days post-spike, and none beyond. Infectious H1N1 LPAIV wasn't isolated from any sediment sample. Our results suggest that slow flowing freshwater surface waters may provide conditions facilitating bird-to-bird transmission for a short period when water temperature are between 10 and 18 °C, though persistence for extended periods (e.g., weeks or months) may be less likely. We hypothesize that experiments simulating real environments, like the one described here, provide a more realistic approach for assessing environmental persistence of AIVs.

1. Introduction

Avian influenza viruses (AIVs) belong to the *Orthomyxoviridae* family and have a segmented, single-stranded RNA genome. AIVs have an evolutionary plasticity that allows them to replicate in different species like waterfowl, poultry, and mammals, including humans. Based on the ability to cause disease and death in chickens, AIVs can be classified into two different pathotypes: low pathogenic (LP) and highly pathogenic (HP). LPAIVs can be asymptomatic, but they typically cause mild to moderate respiratory disease in chickens, often accompanied by a decrease in water or feed consumption and drops in egg production. In contrast, HPAIVs cause severe systemic disease with very high mortality in chickens and other Galliformes (Reperant et al., 2012). Outbreaks of AIV currently represent a big challenge for the poultry sector, wildlife, and public health, as exemplified by the 2021–2022 HPAIV epizootic in Europe (European Food Safety Authority, 2022). Even if there is a low zoonotic risk of the current HPAIV strains circulating in Europe, the emergence of strains with zoonotic potential is an obvious concern, as some HPAIVs have been able to replicate in humans (European Food Safety Authority, 2022).

Wild waterfowl are the main reservoir of AIVs (Pantin-Jackwook and Swayne, 2009); they can disseminate these viruses globally through their migration routes and occasionally spread them to poultry, sometimes with devastating effects (Blagodatski et al., 2021; Fourment et al., 2017; Lycett et al., 2016). Furthermore, migratory waterfowl can also suffer from high mortality rates by HPAIV infections, threatening wildlife conservation (Ramey et al., 2022a). Therefore, the study of waterfowl habitats that intersect different avian migration routes is key to understanding transmission dynamics and, ultimately, designing optimal management strategies. Since transmission of AIV among wild birds occurs mainly via the faecal-oral route through water containing viral particles (Hinshaw et al., 1979, 1980; Stallknecht et al., 2010), environmental transmission through this vehicle may facilitate spill-overs to poultry.

Decades of laboratory-based investigations provide evidence that AIV may remain infectious in water and sediments for extended periods (weeks or months) under experimentally controlled conditions. The role of some abiotic factors is well studied: low water temperatures, neutral pH, and low-salinity water conditions substantially increase AIV persistence (Dalziel et al., 2016; Morin et al., 2018; Pepin et al., 2019). The effect of biotic factors has also been studied, showing that in filtered and sterilized water AIV can persist for longer periods than in untreated water collected in natural sources with the presence of microorganisms (Guan et al., 2009; Keeler et al., 2013; Nazir et al., 2010; Nielsen et al., 2013; Zarkov, 2006; Zhang et al., 2014). Furthermore, wild bird-origin AIVs have repeatedly been isolated (Hinshaw et al., 1979; Ito et al., 1995; Lebarbenchon et al., 2011; Markwell and Shortridge, 1982; Okuya et al., 2015) or detected by RT-PCR (Hénaux et al., 2012; Lickfett et al., 2018) from surface water, and isolated (Poulson et al., 2017) or detected by RT-PCR (Himsworth et al., 2020; Lang et al., 2008) from sediments collected from freshwater and estuarine wetlands. Regarding the amount of AIV needed to initiate an infection in waterfowl via the faecal-oral route, a recent study suggests that even very low titers, around 2 $\log_{10}$ 50 % tissue culture infection dose ($TCID_{50}$) may be sufficient (Ahrens et al., 2022). These findings are in line with the hypothesis that environmental transmission plays a crucial role in AIV epidemiology. However, field experiments carried out under environmental conditions to validate results from laboratory investigations are rare (Reeves et al., 2020). Only recently, a combination of field- and laboratory-based approaches suggested that waterfowl AIVs could remain viable for months in surface water of northern wetlands in North America, supporting the tenet that surface waters may act as an important vehicle in which AIV may be both transmitted and maintained, potentially serving as an environmental reservoir for infectious AIVs (Ramey et al., 2022b). Nevertheless, the effect of UV light and/or water movement were not examined in that study.

Most information about AIV persistence in water has been obtained from studies where AIV was diluted in water and maintained inside a tube (virus-in-a-tube experiments), under controlled conditions lacking the realism of natural ecosystems. Here, we used artificial streams that can mimic biotic and abiotic natural conditions of waterfowl habitats, providing a more refined inference on the duration of AIV infectivity in wetland systems. More specifically, we assessed the effect of temperature (10–13 °C vs. 16–18 °C), the effect of sediments (with the associated microbiota), and the interaction of these two variables on LPAIV water persistence in a more realistic model. Based on previous works, we hypothesized that: 1) colder water temperature (10–13 °C) might have a greater protective effect on infectious viral particles than warmer water temperature (16–18 °C); 2) the presence of sediments could have a sorption effect on AIVs protecting them from abiotic effects; and 3) correspondingly, the interaction of colder water temperature with the presence of sediments could have the main protective effect on AIVs. Given recent HPAIV detections in wetlands from the Mediterranean coast of Catalonia (Ministerio de Agricultura, 2022) and throughout Southern Europe, we also used our results to speculate on AIV persistence in Mediterranean waterfowl habitats during the winter-spring period.

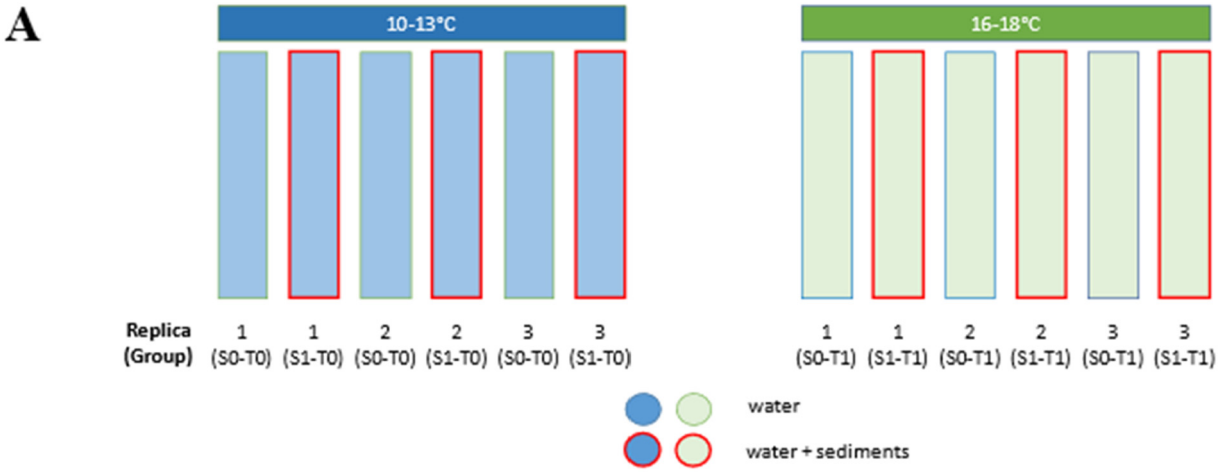
2. Materials and methods

2.1. Virus stock

The LPAIV isolate A/Duck/Italy/281904/06 H1N1 LPAIV was used (kindly provided by Dr. Ana Moreno from the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Italy). The working stock was prepared in embryonated chicken eggs (ECE) (Spackman and Killian, 2014), filtered (0.2 μm), and titrated in Madin–Darby Canine Kidney (MDCK, ATCC CCL-34) cells using the $TCID_{50}$, calculated following the Reed and Muench method (Hierholzer and Killington, 1996).

2.2. Experimental design

The experiment was performed at the artificial streams facility of the Catalan Institute for Water Research (ICRA, Girona, Catalonia, Spain). This indoor experimental system simulates the natural water environment under controlled conditions (Romero et al., 2019). Here, four different treatments were evaluated (three replicates per treatment), with two sediment conditions (presence [S1] vs. absence [S0]) and two temperature conditions (10–13 °C [T0] vs. 16–18 °C [T1]) akin to those found in a Mediterranean river during winter-spring (Fig. 1). Water temperature varied over time following day/night cycles of light, a constant parameter from the experimental set-up, as explained below. Each artificial stream



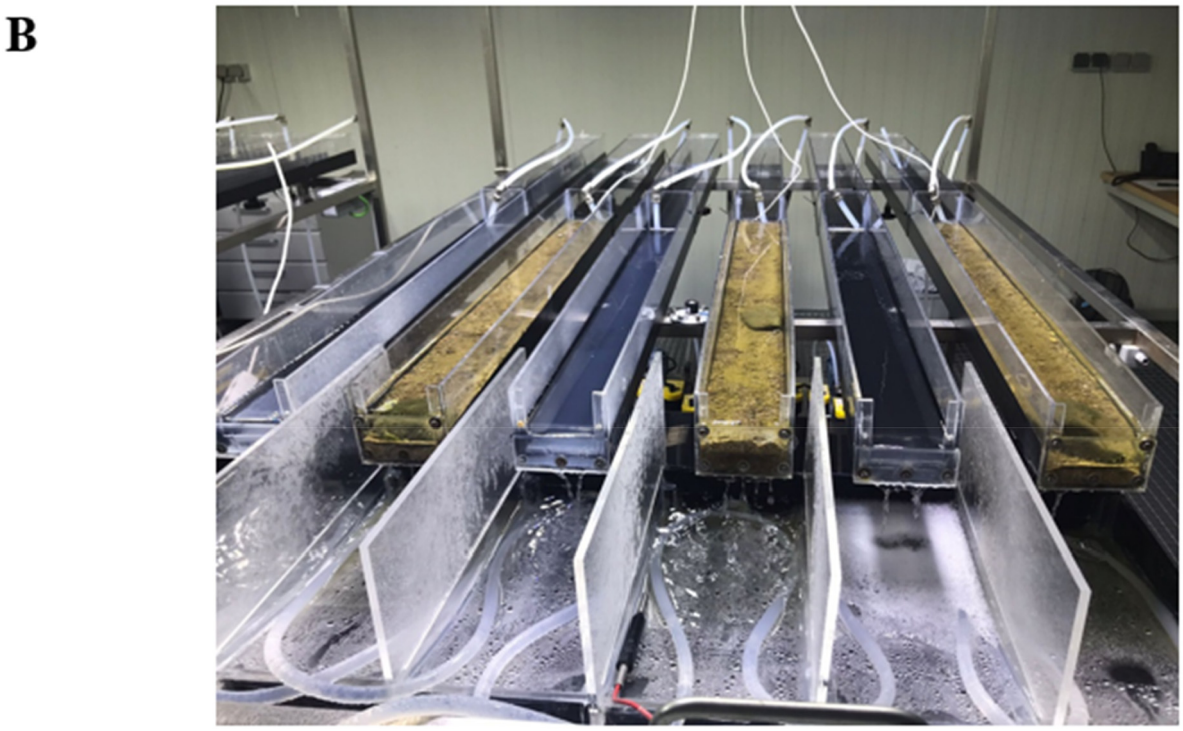


Fig. 1. A. Schematic representation of the artificial streams. B. Artificial streams at ICRA, showing six of the 12 streams used in the experiment.

was 2 m long, had a rectangular cross-section of 50 cm^2 and was set up as an open system with constant slope and steady, uniform flow. Water and sediments were obtained from a non-polluted lower section of the Ter River (Jafre, Catalonia, Spain) close to a wetland area where many species of waterfowl are present (Aiguamolls del Baix Ter, Catalonia, Spain). Sediments were mostly sandy and median sediment grain size was 0.02 mm. Controlled parameters constant to all treatments were daily cycles of photosynthetic active radiation (PAR) (12 h daylight + 12 h darkness) that emulated the solar radiation, reproducing all the wavelengths at the same intensity modifying water temperature, and 5.5 l of water in a recirculating flow of 0.01 l/s. These cycles of photosynthetic active radiation were chosen to mimic autumn day/night cycles in the Mediterranean region, the season when some waterfowl species arrive from their summer breeding areas. The recirculating flow of 0.01 l/s was the lowest flow possible in the artificial streams facility and was chosen to mimic very low flow in wetland areas and to avoid a fast eutrophication process due to still water in the streams. All streams were in acclimation for 3 weeks prior to virus spike, a necessary step for the sediment microbiota to colonize the water. The acclimation process was verified as explained below (Section 2.3). Following acclimation, water and sediment samples from all streams were collected and used as negative controls. Subsequently, all streams were spiked with 53.5 ml of H1N1 LPAIV at a titer of 7.3 $\log_{10}$ $TCID_{50}$ directly into each 5.5 l stream, resulting in a spike dose of approximately 5.3 $\log_{10}$ $TCID_{50}$/ml per stream. The dilution was carried out by gently spiking the virus longitudinally on each stream water surface. Following the virus spike, all streams were sampled and monitored for 14 days as detailed below. Water evaporation was compensated for by adding 400 ml of Ter river water per stream daily. As a positive control, viral persistence was also assessed under virus-in-a-tube conditions at Centre de Recerca en Sanitat Animal (IRTA-CReSA, Bellaterra, Catalonia, Spain). Briefly, 24 Eppendorf tubes (12 tubes at 10 °C and 12 tubes at 16 °C) containing each 1.5 ml of water from Ter River without sediment were spiked with H1N1 LPAIV at a final titer of 5.3 $\log_{10}$ $TCID_{50}$ per ml and maintained at their corresponding temperature for 14 days.

2.3. Characterization of water and sediments

Water and sediment samples were collected throughout the experiment for characterization purposes. Dissolved oxygen, pH, and specific conductivity were measured every 48 h by noon at each artificial stream using Wissenschaftlich-Technische Werkstätten (WTW) hand-held probes (Weilheim, Germany). Water temperature in each artificial stream was recorded every 10 min during all the experiment by means of VEMCO minilog temperature data loggers (TR model; AMIRIX Systems Inc., Halifax Canada) (5–35 °C, ±0.2 °C). Photosynthetically active radiation was also recorded every 10 min using 4 quantum sensors (LI-192SA; LiCOR Inc., Lincoln, NE, USA) located across the whole array of streams.

Biofilm colonization was allowed in the artificial streams for 3 weeks before virus spike. Briefly, superficial sediments (1–10 cm depth) were brought from a nearby lowland river (Ter River near the mouth, Girona), then spread, with no further treatment, in the artificial streams forming a layer of 3–4 cm depth. A colonization period was then allowed so that the biofilms could adapt and grow on the artificial streams. During this acclimation period, biofilm status was monitored twice per week for their maximum photosynthetic efficiency. These measurements provided information on the physiological status of the biofilm (Sabater et al., 2007) in the artificial streams and were made to assess the physiological similarity of the biofilms in all the artificial streams before exposure to the different temperatures. Biofilm samples were collected in streams with sediments after the acclimation period (pre-spike) and at 14 days post-spike at both temperature ranges (see Section 2.6).

2.4. Sampling of water and sediment

Water and sediment samples were collected from all streams at 12 different time-points: pre-spike (as a negative control), 0 (10 min post-spike), 1, 2, 3, 4, 5, 6, 7, 9, 11, and 14 days post-spike. Briefly, 9 ml of water per stream and time-point were collected with a micropipette and stored in 1.5-ml aliquots (Suppl. Photo1) at −75 °C. Approximately 2 ml of sediments per stream and time-point were collected following the "core" method (Suppl. Video 1), with a 15-ml falcon tube and stored at −75 °C. Similarly, one tube per temperature condition and time-point from the virus-in-a-tube experiment was collected and stored at −75 °C.

2.5. Viral detection and quantification in water and sediment samples

Water and sediment samples were used for virus isolation and RNA extraction. While water samples were processed without any additional manipulation, the procedure for extracting virus from sediments was optimized for virus recovery prior to processing samples. To that end, two different methods were tested and compared using sediment samples collected as negative controls from our experiment that were subsequently spiked with 5.3 $\log_{10}$ $TCID_{50}$/ml of sediment with H1N1 LPAIV. For the first method, a sterile gauze was wet with 5 ml of brain heart infusion broth supplemented with 2 % Penicillin/Streptomycin (Invitrogen, Spain). Then the sediment pebbles were manually rubbed with the wet gauze, and the gauze was placed in a sealable plastic bag. A masticator homogenizer (IULmicro, Spain) was used for 1.5 min to punch and squeeze the supernatant out of the gauze and inside the plastic bag. Finally, the supernatant was collected into sterile 50-ml falcon tubes using a micropipette and squeezing the remaining supernatant out of the gauze with a 20-ml syringe. This sediment supernatant was used for virus isolation and RNA extraction alongside water samples. For the second method, the same steps were followed, but a pestle instead of a masticator was used, as previously described (Stephens and Spackman, 2017). The first method using the masticator was able to recover 4.75 $\log_{10}$ $TCID_{50}$, in contrast with the method using the pestle which only recovered 4.2 $\log_{10}$ $TCID_{50}$, validating the use of the method using the masticator.

Water and processed sediment samples were used for quantification of M gene copies. Viral RNA was extracted using Nucleospin RNA virus kit (Macherey–Nagel, Düren, Germany) following manufacturer's instructions. A highly conserved region of 99 bp present in AIV M1 gene was amplified and detected by one-step Taqman real-time RT-PCR (rRT-PCR) in Fast7500 equipment (Applied Biosystems, USA) (Spackman et al., 2002). A standard curve from the same region of the AIV M1 gene was obtained as previously described by our group (Sánchez-González et al., 2020) and used to obtain the gene equivalent copies from the rRT-PCR results. Furthermore, virus isolation from water and sediment samples was conducted in ECEs by standard methods (Spackman and Killian, 2014). Briefly, 3 ECEs were inoculated with 0.2 ml of undiluted water and sediment samples by the chorioallantoic sac route. Standard hemagglutination assay was used to test the allantoic fluid from each ECE for virus replication (Killian, 2014). Hemagglutinating allantoic fluids were assumed to represent the presence of infectious AIV in water and sediment samples.

2.6. Characterization of the sediment microbiota

DNA was extracted from biofilms developed on sediments using the FastDNA Spin for Soil kit (MP Biomedical) according to the manufacturer's instructions. High-throughput multiplexed *16S rRNA* gene sequencing with the Illumina MiSeq System (2 × 250 PE) was carried out using primer pair 515f/806r (Caporaso et al., 2011) targeting the V4 region of the *16S rRNA* gene complemented with Illumina adapters and sample specific barcodes. Details on the analysis of sequence datasets including comparison of alpha and beta diversity of samples across treatments are described in Supplementary Material.

2.7. Statistical analysis

Results obtained from AIV rRT-PCR were used to evaluate the effects of the temperature and the presence or absence of sediments by two methods. First, a Kruskal-Wallis Test (Kruskal and Wallis, 1952) was used to assess differences between groups. If any differences were found, a Dunn's Test (Dunn, 1961) for multiple comparisons was applied to test which treatments were significantly different. Second, to compare the treatments for each time point, a mixed-effects model with the stream as a fixed factor and treatment and time point as random factors was adjusted, along with a contrast of the estimated marginal means. Results obtained from virus isolation were used to determine differences between treatments in their survival curves with a Gehan-Breslow-Wilcoxon test. All the statistical analyses were performed with the R software (R Core Team, 2020) and the subsequent packages: car, FSA, lmerTest, emmeans and tidyverse.

3. Results

3.1. Physical and chemical characterization of water and sediment samples

The temperature of the streams under colder conditions was maintained between 10 °C and 13 °C (mean of 11.6 °C and a standard deviation of 0.95), while the temperature of the streams under warmer conditions was maintained between 16 °C and 18 °C (mean of 17.5 °C and a standard deviation of 0.89) throughout the experiment, temperature variation was due to day/night PAR cycles. The pH in all the streams and time points was around 8, and the specific conductivity was moderate (≈ 500 μs/cm), without significant differences among streams at any time. The physical and chemical characterization of the water did not show significant differences among treatments throughout the experiment (Suppl. File 1).

3.2. Characterization of the sediment microbiota

Characterization of microbial communities in the streams containing sediments was performed to assess potential variations across treatments (i.e., water temperature) that could affect viral persistence during the experimental period. Bacterial communities in sediments did not show significant differences in richness and diversity among streams differing in water temperature (10–13 °C vs. 16–18 °C) (Suppl. Fig. S2). However, a Principal Coordinates Analysis (PCoA) ordination of samples using the Bray-Curtis dissimilarity distance segregated samples according to both the water

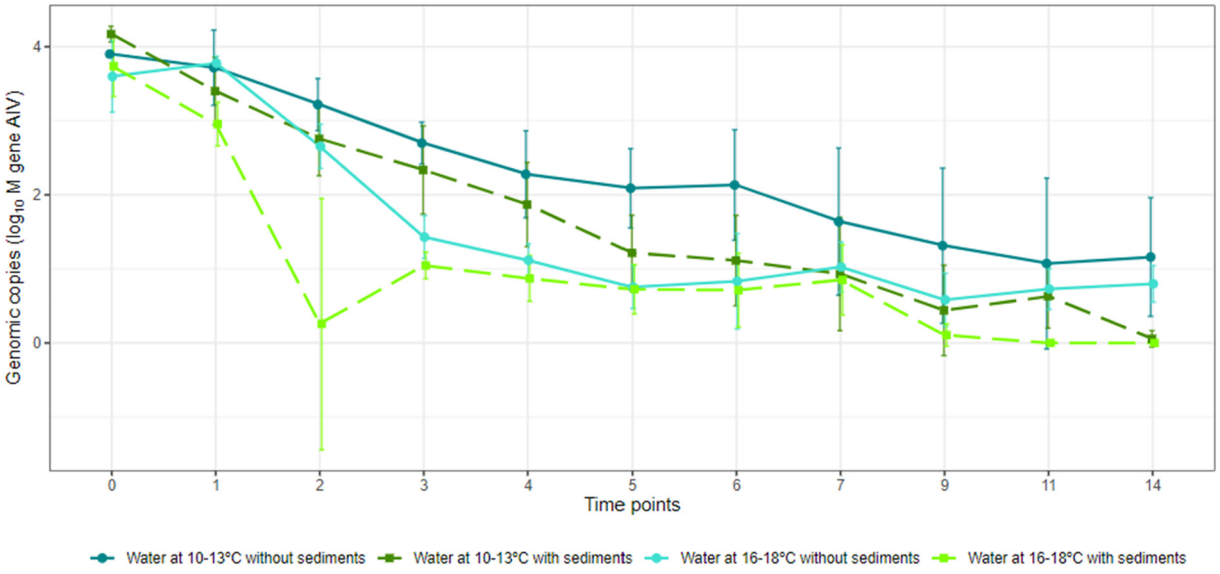


Fig. 2. M gene copies in **water** samples in the four analysed treatments in the artificial streams experiment. Results are expressed as mean and standard deviation (three replicates per treatment) expressed in $\log_{10}$ values.

temperature (horizontal axis, 26 % sample variance, Suppl. Fig. S3) and the experimental time (time 0 vs. time 14 days, vertical axis, Suppl. Fig. S3) (PERMANOVA test, F-value = 2.4207; $p < 0.016$).

3.3. Viral RNA quantification in water and sediment samples

Water and sediment samples from the artificial streams experiment were used for RNA extraction and quantification of H1N1 LPAIV M gene copies by rRT-PCR at 12 different time points. All negative control samples (samples collected pre-spike) were negative, confirming the absence of AIV from water and sediments from Ter River. Overall, water and sediment rRT-PCR results showed presence of viral RNA throughout the 14-day experiment, although a steady decline over time was observed (Figs. 2 and 3).

In water samples, significant differences in viral RNA decay were only observed between streams at different temperatures (10–13 °C vs. 16–18 °C) regardless of sediment condition (multiple comparison Dunn's test). However, the interaction between 10 and 13 °C temperatures and the absence of sediment compared with the interaction of 16–18 °C temperatures and the presence of sediment showed the most significant differences in LPAIV RNA persistence (Suppl. Table S1). In sediment samples, no significant differences in viral RNA decay were observed between streams at different temperatures (Kruskal-Wallis test) (Suppl. Table S2). Significant lower numbers of genomic copies were detected in sediment samples compared to water samples on the first days (taking together both temperature conditions), but similar numbers were found in both types of samples on subsequent days (Fig. 4 and Suppl. Table S2).

3.4. Viral isolation from water and sediment samples

To detect infectious H1N1 LPAIV, viral isolation in ECE from water and sediment samples was carried out. Viral isolation from water samples was successful from all the streams at 0 days post-spike, from six of 12 streams

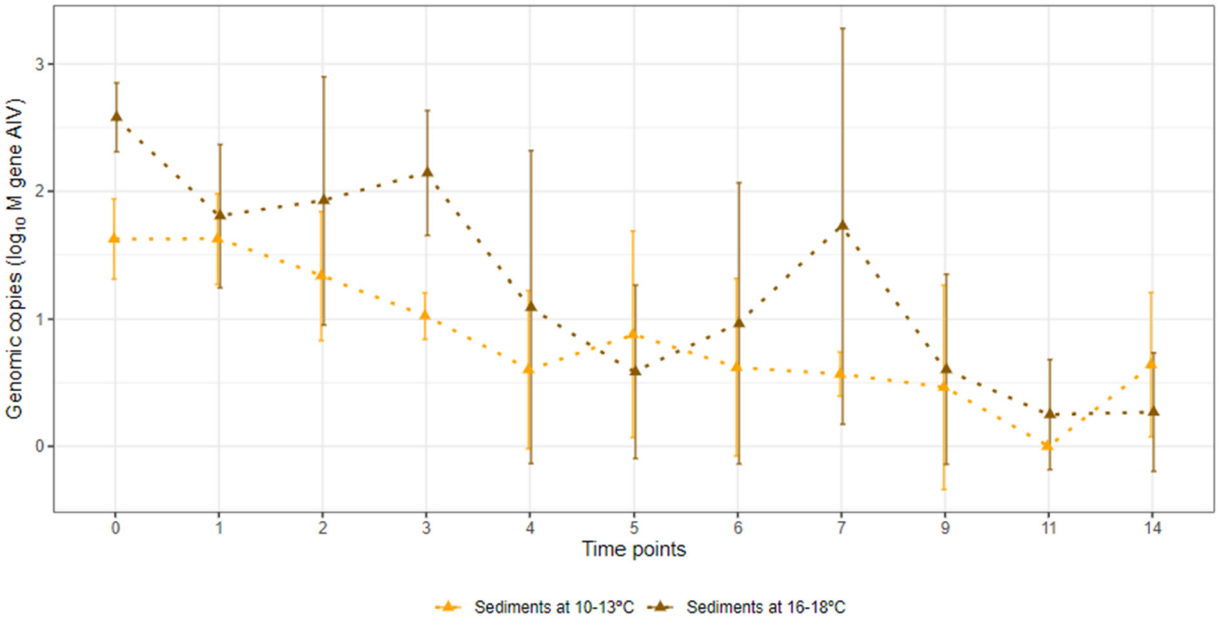


Fig. 3. M gene copies in **sediment** samples in the two analysed treatments in the artificial streams experiment. Results are expressed as mean and standard deviation (three replicates per treatment) expressed in $\log_{10}$ values.

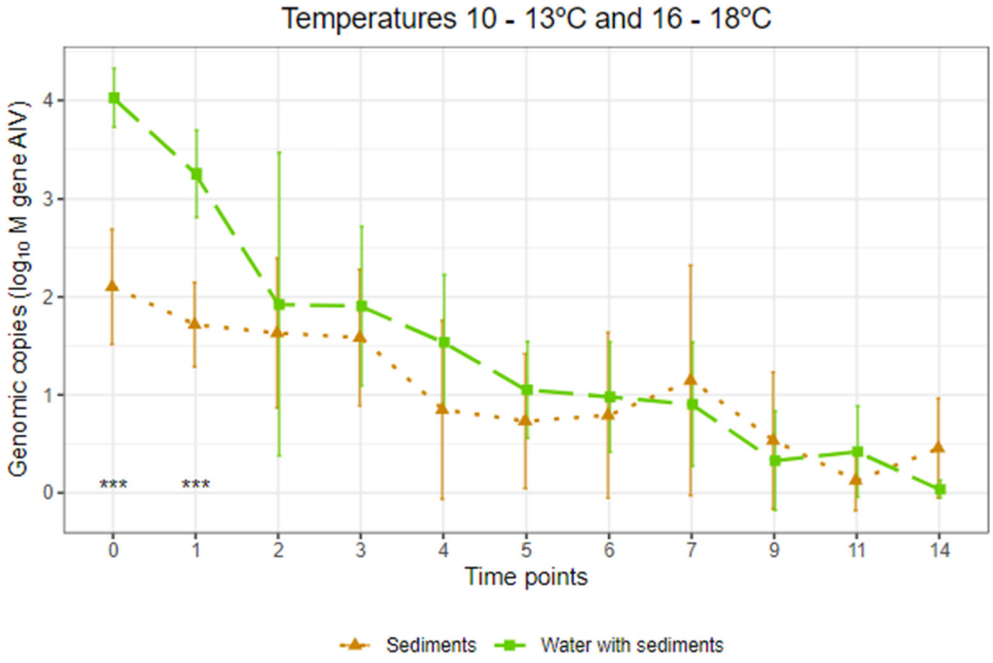


Fig. 4. M gene copies in **sediment samples and water samples** from streams with sediments. Results are expressed as mean and standard deviation (three replicates per treatment) expressed in $\log_{10}$ values.

at 1 day post-spike, from one stream at 2 days post-spike, and no infectious viruses were detected beyond that (Table 1). No significant differences in viral survival among treatments were detected (Table 1). No infectious virus was recovered from any of the sediment samples. In contrast to the artificial streams, viral persistence in water under virus-in-a-tube conditions yielded infectious viruses from all samples at all time points, both at 10 °C and 16 °C temperature conditions (Table 1).

4. Discussion

Most of the data regarding environmental AIV persistence comes from virus-in-a-tube experiments, which do not completely reproduce real environmental conditions (Dalziel et al., 2016; Morin et al., 2018; Pepin et al., 2019). Even if some studies have attempted to reproduce more realistic environmental conditions (Horm et al., 2012; Ramey et al., 2022b), the persistence of AIV in water has not been yet fully characterized in simulated environments. In this study, we mimicked biotic and abiotic natural conditions of waterfowl habitats using artificial streams to analyse the effect of temperature and sediments on the persistence of LPAIV. Even if we still didn't fully characterize the simulated environment, we tried to go one step further mimicking factors such as UV light cycles and streamflow.

First, we characterized the streams after the acclimation period. Similar physical and chemical properties were found among the streams at the same temperature and sediment conditions. Water had a neutral-to-basic pH and moderate specific conductivity throughout all the experiment; these characteristics were previously associated with the longest AIV persistence periods (Dalziel et al., 2016; Keeler et al., 2014; Spackman and Killian, 2014). Furthermore, the characterization of bacterial communities

Table 1
Results of virus isolation from water samples for each replicate by treatment in each of the different experiment setups (i.e., artificial streams and virus-in-a-tube conditions). Results are shown as the number of positive replicates/total replicates. NA = not apply.

Treatment	Sediment	Temperature	Experiment setup	Day 0	Day 1	Day 2	Day 3 to14
S1-T0	Present	10–13 ºC	Artificial streams	3/3	3/3	0/3	0/3
S0-T0	Absent	10–13 ºC	Artificial streams	3/3	1/3	1/3	0/3
S1-T1	Present	16–18 ºC	Artificial streams	3/3	1/3	0/3	0/3
S0-T1	Absent	16–18 ºC	Artificial streams	3/3	1/3	0/3	0/3
NA	NA	10 ºC	Virus-in-a-tube	3/3	3/3	3/3	3/3
NA	NA	16 ºC	Virus-in-a-tube	3/3	3/3	3/3	3/3

in the sediments was also analysed to assess the potential effect of the sediment microbiota on viral persistency. No great differences in richness and diversity of sediment microbial communities were observed across treatments, although beta diversity analyses revealed differences between streams according to water temperature and incubation time. This observation suggests that maturation of biofilm communities was clearly influenced by ambient conditions (i.e., temperature). Recently, Romero and co-workers reported a 92 % similarity between bacterial communities from the artificial streams and those from the source location (Romero et al., 2019). Here, since the conditions of the artificial streams were very similar to those of the source river (non-polluted reach of the river Ter, see Materials and Methods), we can assume that the studied sediment microbiota was analogous to that in the real, streambed biofilms. Overall, our results indicate that the conditions, both abiotic and biotic, in the artificial streams were comparable to those existing in surface water from the area of Spain from which water and sediment was collected and where AIV may persist.

Viral RNA genomic copies throughout the experiment were determined in water and sediment samples by rRT-PCR. It is worth mentioning that we did not concentrate water samples since our goal was to obtain the most realistic results. Genomic copies of the spiked LPAIV were detected both in water and sediment samples until the end of the experiment (14 days), suggesting that the use of environmental samples for viral RNA detection could be a good surveillance method for the early detection of viral RNA from AIVs, as was previously suggested but not to reveal actual infectious AIV virus (Coombe et al., 2021; Hood et al., 2021; Pepin et al., 2019). However, fewer genomic copies were found at day 0 than expected, a phenomenon probably related to viral aggregation after the addition of LPAIV to water at different pH and conductance levels (Floyd and Sharp, 1977, 1978; Kahler et al., 2016; Pradhan et al., 2022). Significant differences in genomic copies in water were observed between streams at different temperature conditions regardless of the sediment condition, but not between streams with or without sediments at the same temperature condition. This observation suggests that, in our study, the presence of sediments —and therefore of associated biotic factors— played a less important role in the degradation or retention of viral RNA than the temperature. These results could be associated with the absence of organisms capable of filtering or feeding LPAIV in our artificial streams (Ma et al., 2021; Meixell et al., 2013; Root et al., 2020). Also, water movement (even if minimal) could be the reason for the low sediment retention. However, a significant combined effect of temperature and sediments in viral decay was observed, with higher viral molecular signal in colder streams without sediments. Furthermore, there was a reduction in the difference of RNA amplification signal between water samples and sediment samples over time, with almost no differences in the last days. These results suggest some degree of viral sorption in sediments. In contrast, and as expected, the temperature had a clear significant effect on viral detection in water samples, with significantly higher amounts of gen amplification signal detected in the colder streams. The favouring effect of cold-water temperature in LPAIV persistence is widely known (Brown et al., 2009; Dalziel et al., 2016; Stallknecht et al., 1990a, 1990b). The lack of significant differences in viral molecular signals in sediment samples between streams at different temperatures could be related to a protective effect of the sediments against abiotic factors, such as temperature.

The ultimate objective of our study was to analyse viral infectivity over time to determine the infectious capacity of LPAIV in Mediterranean waterfowl habitats. For that purpose, water and sediment samples were inoculated in ECE to determine their infectivity. The detection of viral RNA by rRT-PCR did not associate with the recovery of infectious particles in water samples. Even though viral isolation was successful from all water samples at 0 days post-spike (10 min after spike) and from six streams on day 1, isolation in ECE was only successful in one stream at 2 days post-spike. No significant differences in viral isolation results were observed between streams differing in water temperature and presence of sediment, probably due to the rapid decrease in viral infectivity observed in all streams. Surprisingly, we found relatively short viral persistence compared to virus-in-a-tube studies, even though our initial spiking dose was close to other studies (Brown et al., 2009). Consistently, we also found relatively short viral persistence in our artificial streams compared to our virus-in-a-tube experiment, performed as a positive control. Our artificial streams results are in line with a simulated environment study that reproduced real conditions of Cambodian lakes in experimental aquatic biotopes, where LPAIV isolation from water was possible during a maximum of 4 days post-spike (Horm et al., 2012). Nonetheless, another realistic study performed in Alaska showed longer persistence results, with evidence for interannual LPAIV persistence (Ramey et al., 2022b). The differences observed between these simulated environments studies are probably related to differences in water temperature, since higher temperatures were used to reproduce Cambodia's temperatures (25 °C) than Alaska's (0 °C–16 °C), and to the different methodologies used, even if initial spiking doses were like ours. We suggest that abiotic factors such as UV light with the associated variation in water temperature and/or the water movement, which were not incorporated in previous studies (Horm et al., 2012; Ramey et al., 2022b) but were analysed in ours, could be related to our short viral persistence. In fact, UV light, at a specific wavelength, is used as a biosafety technique to inactivate AIV in routine laboratory work (Nishisaka-Nonaka et al., 2018). Furthermore, infectious viruses were not protected inside a tube as in virus-in-a-tube experiments. For these reasons, PAR cycles could have greatly influenced the results. We suggest that northern breeding areas, with very few hours of light during winter and an incidence angle that can make the radiation less penetrating, could reduce the effect of this factor. We also hypothesize that faeces or feathers could protect viral particles against UV light. In any case, further studies need to be done to confirm these hypotheses.

No infectious LPAIV was recovered from any of the sediment samples, suggesting that sediments did not play a major role in viral persistence in our study. However, several studies have described that sediments can act as AIV reservoirs, although viral infectivity was not evaluated (Densmore et al., 2017; Himsworth et al., 2020; Lang et al., 2008) or a *gem* carrier was used (Nazir et al., 2011). In fact, one study tried to isolate infectious virus from sediment samples and was successful only at high infectious doses ($\geq$100 plaque-forming units per 200 μl), linking such low recovery rate from sediment samples to inhibition, disruption of viral particles, or viral attachment to the sediment (Numberger et al., 2019).

Our study was performed in artificial streams mimicking the environmental conditions of the waterfowl habitat in a Southwestern European region with a Mediterranean climate (Catalonia, Spain) during the winter-spring period. In this specific region and conditions, we can find AIV susceptible species, such as the grey heron (*Ardea cinerea*), the yellow-legged gull (*Larus michahellis*), and the mallard duck (*Anas platyrhynchos*) among others (Museu del Ter, 2022). Indeed, outbreaks of HPAIV in waterfowl have already been detected in recent years. For example, in a wetland area from Catalonia (Parc Natural dels Aiguamolls de l'Empordà, Girona, Spain), which is close to the Ter River, HPAIV has been detected in a white stork (*Ciconia ciconia*) during the 2016–2017 season, and in a white stork and a greylag goose (*Anser anser*) during the 2020–2021 season. More recently, seven positive wild birds were detected in wetlands in Catalonia and 38 wild birds all around Spain during the 2021–2022 winter migration period (Ministerio de Agricultura, 2022). Our experimental results corroborate that waterfowl habitats in these mild-temperature latitudes can still provide the right conditions for bird-to-bird (faecal-oral) transmission of AIVs among waterfowl, both migratory and resident, that normally aggregate in a specific area for a short period, as seen in the recent outbreaks with numerous AIV-positive wild birds from the same location. Even if more experiments need to be done, with different water and sediments sources or with different AIV strains, our results seem to suggest that interannual persistence of LPAIV in water ecosystems is unlikely in such Mediterranean climate. This underscores direct or almost direct faecal-oral transmission in contrast to environmental transmission (i.e., without the presence of infected birds), due to the low persistence times found. However, these results could differ significantly in other latitudes with environmental conditions more favourable for AIV persistence. In fact, studies reproducing Alaska's conditions suggest that its water ecosystems play a crucial role in LPAIV transmission among migratory waterfowl, as they are a breeding place for these birds (Ramey et al., 2020,

2022b). This highlights a paradox of our study, while it better approximates real conditions in the field, it also narrows the parameters to which the results can be applied, here restricted to some relevant conditions mimicking a specific region and season. Furthermore, our study did not consider the protective effect of faeces and feathers on LPAIV persistence that some authors suggested (Karunakaran et al., 2019; Nazir et al., 2011) so longer persistence times could be found if this protective effect would have been incorporated. Nevertheless, artificial streams that reproduce waterfowl breeding ecosystems, such as Alaska's, could be used to study the effect of abiotic factors such as UV light, and to confirm the role of these ecosystems in the interannual persistence of LPAIV.

Abbreviations

AIV	avian influenza virus
LPAIV	low pathogenic AIV
HPAIV	highly pathogenic AIV
$TCID_{50}$	50 % tissue culture infection dose
ECE	embryonated chicken eggs
PAR	photosynthetic active radiation
PCoA	Principal Coordinates Analysis
rRT-PCR	real-time RT-PCR

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CRediT authorship contribution statement

The project was conceived, and funding was obtained by MN, AR, and NM. The experiment was designed by AP, KB, VA, MC, FXA, and NM. The experiment was performed by AP, VA, and KB. Samples were collected and processed by AP, KB, VA, and RV. LPG has done the statistical analysis. CMB has done the microbiome analysis of sediments. Figures were prepared by LPG, AP, and KB. The manuscript was drafted by AP with corrections from MC, KB, VA, FXA, MN, AR, LPG, and NM. All authors contributed to the article and approved the submitted version.

Data availability

Data will be made available on request.

Declaration of competing interest

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

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