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Experimental evaluation of *Haplosporidium pinnae* DNA detection and degradation under semi-controlled conditions

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Abstract

The fan mussel *Pinna nobilis* is one of the most endangered marine species in the Mediterranean Sea, having suffered mass mortality events driven primarily by the haplosporidian parasite *Haplosporidium pinnae*. To support the conservation of this critically endangered bivalve, the development of reliable, non-invasive detection methods is essential. Here, we tested the persistence of *H. pinnae* DNA in seawater under semi-controlled aquarium conditions through two filtration experiments. In the first experiment, seawater was enriched with parasite DNA from a live infected individual prior to its removal, whereas in the second experiment, enrichment included post-mortem release. Water samples were collected over several weeks and tested using end-point PCR and real-time PCR assays targeting *H. pinnae*. Parasite DNA was detectable for up to 18 days in the live-infected experiment and 15 days in the post-mortem experiment. Detection success depended strongly on amplicon size, with shorter fragments persisting longer than larger ones. Real-time PCR also revealed different degradation dynamics between the two experiments, suggesting variation in the initial DNA load and release mechanisms. These results provide an empirical baseline for understanding the persistence of *H. pinnae* DNA in seawater. Although environmental variability in open waters may shorten detection windows, our findings demonstrate that eDNA remains detectable long enough to inform monitoring efforts. From a conservation perspective, eDNA assays could help identify *H. pinnae*-free refugia and support proactive strategies for safeguarding surviving populations of *P. nobilis* across the Mediterranean.

Keywords: *Pinna nobilis*; noble pen shell; critically endangered; protozoan parasite; environmental DNA; non-invasive monitoring.

List of abbreviations: bp – base pairs; DNA – deoxyribonucleic acid; eDNA – environmental DNA; GF – glass fibre filter; MCE - cellulose mixed ester filter; MME – mass mortality event; PCR – polymerase chain reaction; rRNA – ribosomal ribonucleic acid

Introduction

Pinna nobilis (Linnaeus, 1758), commonly known as noble pen shell or fan mussel, is a large, filter-feeding bivalve endemic to the Mediterranean Sea. While commonly reaching between 20 and 80 cm in length (Poppe & Goto, 1993), it can grow to over a meter (Vicente & Moreteau, 1991; Zavodnik *et al.*, 1991), playing an important role in maintaining the oligotrophy and cleanliness of the sea by filtering large amounts of water (Trigos *et al.*, 2014). Due to anthropogenic impacts, fan mussel populations severely declined in the twentieth century (Deudero *et al.*, 2015; Richardson *et al.*, 2004), so they fell under the protection of the European Union Habitats Directive (EU, 1992) and also as an endangered species by the Barcelona Convention (UNEP, 1995).

In 2016, mass mortality events (MMEs) of fan mussel populations were reported in Spain (Vázquez-Luis *et al.*, 2017), and in the following few years, MMEs spread across the Mediterranean basin (Cabanellas-Reboredo *et al.*, 2019; Čižmek *et al.*, 2020; Katsanevakis, 2019; Panarese *et al.*, 2019), extending as far as the Black Sea (Künili *et al.*, 2021). Due to these MMEs, the International Union for Conservation of Nature Red List currently lists *P. nobilis* as "Critically Endangered" with a declining population trend (Kersting *et al.*, 2019). Results from the current

LIFE PINNARCA project (LIFE20-NAT/ES/001265) on the conservation of this species across the Mediterranean show a continuation of the declining population trend. Mortalities were quickly associated with the infection by *Haplosporidium pinnae*, a protozoan parasite (Catanese *et al.*, 2018; Darriba, 2017), and although other authors have described co-infections with other pathogens (Box *et al.*, 2020; Carella *et al.*, 2020; Lattos *et al.*, 2020; Prado *et al.*, 2020b; Šarić *et al.*, 2020; Scarpa *et al.*, 2020), *H. pinnae* was later proven to be the main factor responsible for the observed die-off (Grau *et al.*, 2022; Tiscar *et al.*, 2022). More recently, the potential immunosuppressive effect of a picornavirus pathogen in *P. nobilis* has been investigated (Carella *et al.*, 2023). In recent years, pen shell mortality has also reached areas that were previously considered more protected due to existing physical or salinity barriers, such as marine lagoons and regions not directly exposed to the open sea (Çinar *et al.*, 2021; Donato *et al.*, 2023; Foulquie *et al.*, 2023; Künili *et al.*, 2021; Labidi *et al.*, 2023; Nikolaou *et al.*, 2024; Papadakis *et al.*, 2023).

Several molecular methods have been developed and used for detecting the presence of haplosporidian DNA, enabling identification at both the genus level (Renault *et al.*, 2000) and the species level (Catanese *et al.*, 2018; López-Sanmartín *et al.*, 2019; Manfrin *et al.*, 2023; Panarese *et al.*, 2019). While most diagnostics of *H. pinnae* were performed on tissue samples, a recent study examined whether the pathogen can be detected in water samples (Moro-Martínez *et al.*, 2023). Even though the results varied mostly by the source of the water sample, the study showed that *H. pinnae* could be detected using environmental DNA (eDNA).

eDNA-based methods allow the detection of an organism's DNA from various cells or extracellular DNA shed into its surroundings, without the need to capture the target species at any stage of its life (Ficetola *et al.*, 2008). This approach is effective for the early detection of endangered, elusive, endemic, invasive and non-native species (Baudry *et al.*, 2021; Belle *et al.*, 2019; Dubreuil *et al.*, 2022; Piggott, 2016; Vucić *et al.*, 2023), but also for pathogen detection (Bass *et al.*, 2015, 2023; Sieber *et al.*, 2020).

To determine the potential distribution of the parasite, concern should not be limited to the presence/absence but also the possible spread. Determining the spread of pathogens in a marine environment is highly complex (Behringer *et al.*, 2018; Kough *et al.*, 2015; McCallum *et al.*, 2003) and depends on the time the pathogen can survive without its host. In tissues, *H. pinnae* proliferates into spores, found mostly in the digestive gland of the fan mussel (Grau *et al.*, 2022; Panarese *et al.*, 2019), and is probably dispersed by surface currents (Cabanellas-Reboredo *et al.*, 2019). Given the lack of information on the viability and survival of *H. pinnae* spores, the time to detect *H. pinnae* DNA once removed from its source serves as a proxy for the survival duration of *H. pinnae*.

As there is an urgent need to preserve *P. nobilis* populations, conservation efforts have included rescue programs, the installation of larvae collectors, and the protection of infected adults from predators (García-March *et al.*, 2020). Additionally, the development of *ex situ* maintenance protocols for *P. nobilis*, involving the care of individuals in controlled tank environments to promote their growth, rehabilitation, and potential reproduction in pathogen-free conditions, is seeing steady progress (Hernandis *et al.*, 2023; Prado *et al.*, 2020a).

For that purpose, the Noble Sanctuary was established in Aquarium Pula in June 2019 to support the conservation of *P. nobilis*. The Noble Sanctuary is a quarantine unit designed to house *P. nobilis* individuals under controlled conditions for extended periods. The early care experiments provided crucial experience in multiple techniques, such as managing water quality in a closed-circulation system with continuous filtration and sterilisation, temperature regulation, food production, nutrition optimisation and molecular diagnostics of *H. pinnae*.

This study aimed to explore the duration over which *H. pinnae* DNA can be detected in seawater after the separation of pretreated seawater from diseased *P. nobilis*, which act as the pathogen's only source of transmission. To achieve this, we evaluated the degradation of the detected DNA at different time points using molecular methods to target 18S rRNA gene fragments of varying sizes in controlled experiments.

Materials and Methods

Experiment setup

To investigate how long *H. pinnae* DNA remains detectable in seawater once the source organism is removed, we conducted two filtration experiments under semi-controlled aquarium conditions**.** In the Aquarium, all adult individuals of *P. nobilis* are kept separately, each in a 300-L maintenance tank (Fig. 1). Ordinarily, the seawater used for the maintenance tanks is pretreated by circulating it for 24 h through a series of four polypropylene filters with pore sizes of 100, 50, 20 and 1 µm (USTM, Poland) and a T5 UV steriliser type 801 (Deltec, Germany). Then, upon arrival at the Aquarium, adult pen shells are tested for the presence of the *H. pinnae* parasite from a small piece of mantle tissue using the molecular methods described in Catanese *et al.* (2018). One adult *P. nobilis* (52 cm in height, 18 cm in width) collected from the Venice lagoon and transferred to Aquarium Pula on 19 February 2024, tested positive for *H. pinnae* and displayed delayed responses to external stimuli, and was therefore selected for the first filtration experiment. Under standard aquarium conditions, tank water is replaced every 24 h. To test for the presence of *H. pinnae*, the water in which this individual had been kept for 24 h was first sampled, filtered, and analysed by real-time PCR. Because the results were ambiguous, the diseased mussel was maintained in the same water for 72 h to increase the parasite concentration. The water was then transferred to a clean tank, and the routine 24-h water replacement regime for *P. nobilis* was resumed. The transferred water was kept at room temperature (18-21°C) and sampled with Erlenmeyer flasks on the same day (day zero) and on days 1,

Fig. 1: Adult *Pinna nobilis* in a long-term maintenance tank in Aquarium Pula.

2, 3, 4, 9, 10, 11, 14, 15, 16, 17, 18, 21, 22 and 23 after the transfer. Prior to sampling, the water in the tank was stirred with a plastic stick for approximately one minute, and then sampled from the bottom of the tank. For the entire duration of the experiment, except during stirring, the tank was covered to keep the water in darkness. Each sample was filtered immediately after sampling. Sampling flasks were disinfected in an overnight bath of 10% sodium hypochlorite and thoroughly rinsed with ultrapure water produced using the Halios system (Neptec, Germany). This setup ensured that any *H. pinnae* DNA detected originated from the infected mussel and enabled us to track its persistence in the water over time.

After the death of the infected individual (16 June 2024), a second filtration experiment was performed in a similar manner to verify the results of the first experiment. Once it was determined that the individual did not respond to external stimuli, it was left in the same water for one more day, bringing the total time after the water rechange to 48 h. The dead pen shell was then removed from the tank. The water was kept in the same tank and in the same conditions as during the first experiment, and was sampled on the day the pen shell was removed (day zero) and on days 1, 2, 5, 6, 7, 8, 9, 12, 13, 14, 15 and 16 after the removal of the pen shell. Since, in the first experiment, seawater was enriched with parasite DNA using a live infected mussel before its removal, while the second experiment involved post-mortem release, we will refer to them as the live-infected experiment and the post-mortem experiment, respectively.

Filtration and DNA extraction

The water samples were filtered using a Rocker 300 vacuum pump (Rocker, Taiwan) equipped with a Nalgene reusable bottle top filter (Thermo Fisher Scientific, USA). For the first five days of the live-infected experiment, the filtrations were performed using two types of membrane filters: EO-treated glass fiber (GF) filters (Macherey-Nagel, Germany) with a diameter of 45 mm and PORAFIL MV cellulose mixed ester (MCE) membranes reinforced with a polyester fabric with a diameter of 47 mm and a pore size of 0.8 µm (Macherey-Nagel, Germany). Given that the quality of the DNA extracted from the GF filters was poor, the samples were filtered only using the mixed cellulose filters from day 9 of the live-infected experiment and in the post-mortem experiment. The total volume of filtered water was 8 L for the GF filters and 2 L for the MCE filters. This method was chosen because once the filtration approached these volumes, it became extremely slow, even at the highest pressure, with a flow rate of one drop every three to five seconds. After filtration, the filter was put in the sterile 2-mL tube and frozen at -20°C overnight. To avoid potential contamination and cross-contamination between samples, the filters were always handled with tweezers, and after each filtration, the bottle top filter and tweezers were disinfected in a 10% sodium hypochlorite bath for at least 20 minutes and thoroughly rinsed with ultrapure water.

The following day, half of the filter was cut into small pieces, placed into an innuSPEED Lysis Tube A (Innuscreen, Germany) and homogenised on the SpeedMill PLUS (Analytik Jena, Germany) for two 1-min intervals. The filter was handled with tweezers, and the tweezers and scissors were disinfected as above. DNA extraction was performed using the innuPREP DNA Kit - IPC16 (Innuscreen, Germany) with some modifications. After a brief centrifugation, 400 µL of GRS PCR grade water (Grisp, Portugal), 400 µL of Lysis Solution CBV and 40 µL of Proteinase K were added to the tube, and the tube was again subjected to homogenisation for two 1-min intervals. The tube was then incubated in the thermo-shaker for 3 h at 55°C and 600 rpm. After incubation, the tube was centrifuged at 10,000 rcf for 2 min, and 400 µL of the sample was transferred to the reaction plates. The remaining extraction was performed on the InnuPure C16 touch (Analytik Jena, Germany) using the manufacturer's protocol with an elution volume of 50 µL. The quantification and quality control of the extracted DNA were performed using the DS 11 FX spectrophotometer (DeNovix, USA). The measurements of all samples were conducted in triplicate, and the mean concentration and absorbance ratios $A_{260/230}$ and $A_{260/280}$ were calculated from the obtained values for each sample. The extracted DNA was stored at -20°C.

Molecular assays

Three different assays to test for the presence of haplosporidian DNA were performed on all samples: two

end-point PCRs using genus-specific primers HAP-F1 and HAP-R2 (Renault *et al.*, 2000) and species-specific primers HPNF3 and HPNR3 (Catanese *et al.*, 2018), and one real-time PCR using species-specific primers HpF3 and HpR3 (López-Sanmartín *et al.*, 2019). All primers were manufactured by Macrogen Europe. These assays were chosen because they all target fragments of the same gene, but with different lengths of the fragment. The target sizes of species-specific end-point PCR, genus-specific PCR and real-time PCR are 600, ~ 330 and 165 base pairs, respectively.

Both end-point PCR reactions were performed on a Biometra TAdvanced Twin 48 system (Analytik Jena, Germany) using GoTaq G2 Hot Start Colorless Master Mix (Promega, USA) in a total volume of 25 µL per reaction, consisting of 12.5 µL of 2X Master Mix, 0.4 µM of each primer, ~ 200 ng of extracted total DNA and nuclease-free water. The temperature protocol applied was 95°C for 2 min, followed by 40 cycles of 95°C for 30 s, annealing temperature for 30 s and 72°C for 30 s, and ending with a final elongation step at 72°C for 5 min. The annealing temperature was 48°C with HAP-F1/HAP-R2 primers and 55°C with HPNF3/HPNR3 primers. PCR products were visualised using electrophoresis (for details, see Appendix).

Real-time PCR was performed on a qTOWERiris system (Analytik Jena, Germany) using GoTaq qPCR Master Mix (Promega, USA) in a total volume of 20 µL per reaction, consisting of 10 µL of 2X Master Mix, 0.4 µM of each primer, ~ 100 ng of total extracted DNA and nuclease-free water. The temperature protocol applied was 95°C for 2 min, 40 cycles of 95°C for 15 s and 60°C for 1 min, followed by the determination of the melting curve starting at 60°C and ending at 95°C with a 0.5°C increment. For each sample, real-time PCR was performed in three technical replicates.

All molecular assays included three controls: a negative control, no template control and a positive control. To prepare the negative control, 2 L of seawater was taken from a tank with another noble pen shell kept at the Aquarium that tested negative for the presence of *H. pinnae* DNA. This water was filtered using MCE filters, and the DNA was extracted from it as described above. For the no-template control, the reactions were prepared using PCR grade water instead of template DNA. For the positive control, DNA extracted from the tissue of a dead juvenile *P. nobilis*, previously confirmed to be positive for the presence of *H. pinnae* DNA by PCR and sequencing was used. For real-time PCR, all controls were used in three technical replicates.

Results

The water sample filtered with MCE filters after 24 h exposure to the infected *P. nobilis* showed no amplification of *H. pinnae* DNA with either the HAP-F1/HAP-R2 or HPNF3/HPNR3 primers. With the real-time PCR assay, the reactions were positive, with an average C_t of 32.28 and the melting peak at 83.8 °C.

Total DNA extracted from GF filters in the live-infected experiment was of high concentration, averaging 238.6 ± 14.43 ng/µL (Table 1), but of low purity with $A_{260/230}$ ratios ranging from 0.7 to 1.3. All PCR assays using DNA extracted from GF filters were negative.

MCE filters also yielded good total DNA concentrations, ranging from 73 to 290 ng/µL in the live-infected experiment (Table 1) and from 53 to 123 ng/µL in the post-mortem experiment (Table 2). The purity of DNA extracted from MCE filters was higher than that from GF filters, with the $A_{260/230}$ ratios ranging from 1.7 to 2.2. In the live-infected experiment, a progressive increase in the concentration of extracted DNA was observed over time using MCE filters, suggesting a continuous accumulation of genetic material in the samples.

Using a species-specific end-point PCR assay, the presence of *H. pinnae* DNA was detected for up to 11 days in the live-infected experiment (Table 1) and for up to nine days in the post-mortem experiment (Table 2). The use of non-specific primers, which amplify a smaller DNA fragment, enabled the detection of the parasite for up to 15 and 12 days in the live-infected and post-mortem experiment, respectively, extending the period during which its presence could be identified.

Using a real-time PCR assay, *H. pinnae* DNA was detected for up to 18 days in the live-infected experiment (Table 1), though the amplification was positive in only one technical replicate on day 16, two technical replicates on day 17 and a technical replicate on day 18. On other days, all three technical replicates yielded positive amplification. C_t values were relatively high on day zero (25.46 ± 1.65), but over the next four days increased, on average, by 2.61 cycles per day, indicating a sharp decline of the target concentration. From day 4 until day 11 of the experiment, there was almost no change in the C_t value, and then it increased slightly until day 16.

In the post-mortem experiment, real-time PCR was positive up to day 15 (Table 2). All three technical replicates were positive on most days, except on days 6 and 8, when only 2 replicates were positive. C_t values at the start were higher than in the live-infected experiment (32.41 ±1.04). Even though there was a constant daily increase in C_t values, the sharp decline in target concentration over the first few days detected in the live-infected experiment was not observed here. Only between days zero and one did the average C_t increase by 2 cycles (1.97), while on all other days the increase in average C_t ranged from 0.07 to 0.82.

Discussion

This study evaluated the persistence and detectability of *Haplosporidium pinnae* DNA in seawater under semi-controlled aquarium conditions using eDNA-based assays. After removal of the infected host, all subsequent detections reflected DNA already released into the water. Consistent PCR positivity during the early sampling days indicates sufficiently high initial eDNA concentrations for reliable amplification. However, detection of parasite

Table 1. Results of the live-infected filtration experiment. PCR – polymerase chain reaction, bp – base pairs, DNA – deoxyribonucleic acid, MCE – cellulose mixed ester filter, GF – glass fibre filter; +/- – result of the PCR assay, + positive, - negative; C_t – threshold cycle, SD ± – standard deviation, X/3 – how many of the three technical replicates were positive, T_m – temperature of the peak of the melting curve.

	Experiment 1			Conventional PCR		real-time PCR			
				HAP-F1/ HAP-R2 ~ target size 330 bp	HPNF3/HPNR3 ~ target size 600 bp	HpF3/HpR3 ~ target size 165 bp			
Filter	Day	DNA concentration (ng/µL)	DNA purity (A260/230, A260/280)	+/-	+/-	C_t	SD ±	X/3	T_m (°C)
MCE	0	73	(1.9, 2.2)	+	+	25.46	1.65	3	83.8
MCE	1	99	(2.0, 2.1)	+	+	27.42	1.85	3	83.8
MCE	2	90	(1.9, 2.0)	+	+	30.08	2.35	3	83.8
MCE	3	85	(2.0, 2.2)	+	+	32.26	1.05	3	83.8
MCE	4	103	(2.2, 2.2)	+	+	35.88	1.08	3	83.8
MCE	9	133	(2.1, 2.2)	+	+	35.20	1.13	3	83.8
MCE	10	147	(2.0, 2.1)	+	+	35.74	1.77	3	83.8
MCE	11	190	(2.1, 2.2)	+	+	35.62	0.63	3	83.8
MCE	14	133	(1.8, 2.2)	+	-	36.39	1.15	3	83.8
MCE	15	163	(1.8, 2.1)	+	-	37.48	0.15	3	83.8
MCE	16	230	(1.8, 2.1)	-	-	38.83	-	1	83.8
MCE	17	290	(1.7, 2.1)	-	-	37.66	1.94	2	83.8
MCE	18	247	(1.8, 2.1)	-	-	38.02	-	1	83.8
MCE	21	147	(1.8, 2.0)	-	-	-	-	0	-
MCE	22	263	(1.7, 1.9)	-	-	-	-	0	-
MCE	23	243	(1.7, 1.8)	-	-	-	-	0	-
GF	0	264	(0.7, 1.6)	-	-	-	-	0	-
GF	1	229	(1.1, 1.7)	-	-	-	-	0	-
GF	2	244	(1.0, 1.7)	-	-	-	-	0	-
GF	3	233	(1.3, 1.9)	-	-	-	-	0	-
GF	4	223	(1.2, 1.8)	-	-	-	-	0	-

Table 2. Results of the post-mortem filtration experiment. PCR – polymerase chain reaction, bp – base pairs, DNA – deoxyribonucleic acid, MCE – cellulose mixed ester filter; +/- – result of the PCR assay, + positive, - negative; C_t – threshold cycle, SD ± – standard deviation, X/3 – how many of the three technical replicates were positive, T_m – temperature of the peak of the melting curve.

Experiment 1				Conventional PCR		real-time PCR			
				HAP-F1/ HAP-R2 ~ target size 330 bp	HPNF3/HPNR3 ~ target size 600 bp	HpF3/HpR3 ~ target size 165 bp			
Filter	Day	DNA concentration (ng/µL)	DNA purity (A260/230, A260/280)	+/-	+/-	C_t	SD ±	X/3	T_m (°C)
MCE	0	107	(2.2/2.2)	+	+	32.41	1.04	3	83.8
MCE	1	73	(2.1/2.1)	+	+	34.38	0.73	3	83.8
MCE	2	123	(2.1/2.2)	+	+	35.20	1.63	3	83.8
MCE	5	83	(2.0/2.2)	+	+	35.34	1.19	3	83.8
MCE	6	70	(2.0/2.2)	+	-	35.68	0.91	2	83.8
MCE	7	70	(2.0/2.3)	+	+	36.16	0.43	3	83.8
MCE	8	87	(2.0/2.3)	+	-	36.23	1.13	2	83.8
MCE	9	57	(1.9/2.2)	+	+	36.63	1.63	3	83.8
MCE	12	93	(2.1/2.3)	+	-	37.13	0.33	3	83.8
MCE	13	63	(2.0/2.0)	-	-	37.60	1.69	3	83.8
MCE	14	100	(2.2/2.2)	-	-	37.80	2.13	3	83.8
MCE	15	80	(2.0/2.0)	-	-	38.58	1.08	3	83.8
MCE	16	53	(2.2/2.2)	-	-	39.14	-	1	77.6

DNA does not confirm the presence of viable or infectious parasites.

Comparison of the two filtration experiments revealed both shared and distinct patterns. In both cases, *H. pinnae* DNA remained detectable for more than a week, with shorter fragments persisting longer than larger ones. Detection lasted up to 18 days in the live-infected experiment and 15 days post-mortem, with sharper early C_t increases in the former, consistent with a higher initial eDNA concentration. Differences in starting parasite load, post-mortem DNA release, or microbial activity may explain these patterns, underscoring the need for replication when assessing DNA degradation dynamics.

Progressive DNA degradation ultimately reduced detectability, with longer amplicons failing first and shorter fragments remaining amplifiable the longest. Random negative PCRs on certain days likely reflected stochastic sampling, localized degradation, or PCR inhibition. These patterns are consistent with known mechanisms of DNA decay in aquatic environments, where enzymatic activity, microbial degradation, and abiotic factors progressively fragment genetic material (Collins *et al.*, 2018; Strickler *et al.*, 2015; Wood *et al.*, 2020). In the live-infected experiment, a gradual increase in total DNA concentration on MCE filters may indicate increasing bacterial load over time within the sampled water.

These results confirm that *H. pinnae* eDNA has a limited detection window under controlled conditions, with detectability depending on fragment size and assay sensitivity. We increased parasite concentration by prolonging host exposure, but the exact initial overload was unknown, so experimental control was only partial. Compared to the natural environment, key physical (temperature, salinity, hydrodynamics) and biological (microbial communities, organic input) parameters were stabilised, allowing controlled testing of eDNA persistence but limiting direct extrapolation to field conditions. A similar parasite, *H. nelsoni*, is strongly influenced by temperature and salinity (Arzul & Carnegie, 2015), and comparable effects are likely for *H. pinnae* (Cabanellas-Reboredo *et al.*, 2019). Future studies should consider seasonal variation in parasite shedding to optimise field sampling strategies.

None of the assays produced amplicons when GF filters were used, despite their design for eDNA retention (Macherey-Nagel). Similar issues have been reported for other aquatic parasites (Sieber *et al.*, 2020). Likely explanations include the concentration of PCR inhibitors present in bivalves (Schrader *et al.*, 2012) as larger water volumes were filtered, greater water retention within GF filters leading to co-extraction of inhibitors, or suboptimal compatibility between the extraction protocol and GF material. Since MCE filters yielded consistent amplifications, optimising methods for GF filters was considered beyond the scope of this study.

Both experiments demonstrated that eDNA-based molecular methods can be effectively applied to diagnose *H. pinnae* in housed *P. nobilis*, reducing the need for invasive procedures. Similar eDNA approaches have already been used to identify juvenile pen shells when morphological distinction between *P. nobilis* and *P. rudis* is not possible (Catanese *et al.*, 2022). Before the emergence of *H. pinnae*, transplantation of *P. nobilis* was already used as a conservation measure (Bakran-Petricioli *et al.*, 2019; Katsanevakis, 2016), and in recent years, the housing of individuals for research and long-term maintenance has become integral to several conservation initiatives (e.g., LIFE PINNARCA). Assessing the health status of each individual is now essential, and minimising handling and invasive sampling can help reduce stress and improve animal welfare.

As shown in previous work (Moro-Martínez *et al.*, 2023), assays targeting different fragment sizes can be used to track *H. pinnae* DNA degradation over time. Reported degradation rates in marine environments vary widely, from rapid (Collins *et al.*, 2018) to slow (Strickler *et al.*, 2015), depending on factors such as temperature, UV exposure, pH, and sample origin (inshore vs. offshore). The presence of non-target eDNA can also influence detection times (Wood *et al.*, 2020). These observations highlight the need to optimise eDNA protocols for *H. pinnae* under both controlled and field conditions.

The mechanisms underlying *H. pinnae* transmission in a marine environment remain unclear. A spatially explicit model for parasite-driven epidemics in immobile hosts (Giménez-Romero *et al.*, 2021, 2022) predicts that higher parasite mobility accelerates spread and extinction, whereas limited mobility confines infections locally. Accordingly, environmental persistence depends on parasite mortality and diffusion. Our findings of detectable DNA over a limited period under controlled conditions are consistent with the idea that *H. pinnae* or its genetic material can persist long enough to support local transmission, and they also align with model predictions that restricted dispersal can reduce epidemic extent.

Direct transmission likely contributes to the spread of *H. pinnae*, particularly in dense mollusc populations where outbreaks often follow summer currents. Faecal shedding by infected *P. nobilis* is a plausible environmental pathway: expelled spores could remain viable for some time and infect new hosts through filtration. Another possibility involves intermediate hosts, such as small invertebrates or protozoa, which may ingest spores and subsequently transmit the parasite. Spores might also settle in sediments and infect benthic molluscs. Our observation of the persistence of *H. pinnae* DNA indicates that genetic material remains in the water long enough to overlap with these transmission routes, even if viability is not confirmed. Detection of the parasite in faeces has been documented (Lopez-Nuñez *et al.*, 2022; Manfrin *et al.*, 2023). Interestingly, healthy populations in estuarine and lagoon environments suggest that transmission may be less effective there (Nebot-Colomer *et al.*, 2022), possibly due to temperature and salinity differences (García-March *et al.*, 2020). The limited persistence window we observed could partly explain the reduction of transmission pressure in such environments, as environmental factors may further accelerate degradation.

Environmental factors strongly influence the distribution of *Haplosporidium* species, with salinity playing a

key role in *H. pinnae* persistence and transmission (Albuixech-Martí *et al.*, 2020; Prado *et al.*, 2021). Mortality of *P. nobilis* varies with salinity, and transmission is predicted to be most effective between 36.5-39 (Cabanellas-Reboredo *et al.*, 2019). Under less favourable conditions, faster DNA degradation may reduce parasite survival. While our experiments were conducted under aquarium conditions, applying these findings to open-water environments requires caution. In nature, factors such as temperature, salinity, microbial activity, UV radiation, and hydrodynamics can accelerate DNA degradation or dilute it below detection limits, shortening the effective detection window. Nevertheless, these results provide a valuable baseline for understanding *H. pinnae* DNA persistence, which can be tested across environmental gradients, and can inform survey design, help identify parasite-free refugia, and support conservation measures for *P. nobilis*.

Understanding the dynamics of *H. pinnae* DNA degradation is crucial for managing remaining *P. nobilis* populations in the wild. Although assays targeting different fragment sizes can approximate the status of DNA degradation (Moro-Martínez *et al.*, 2023), they do not directly measure parasite viability, so results must be interpreted with caution. Still, for conservation decisions, any positive assay, regardless of amplicon size, should be treated as evidence of parasite presence, even if viability cannot be confirmed. The detection limit of the most sensitive of the three methods used was 30 copies of the 18S rRNA gene per ng of DNA (López-Sanmartín *et al.*, 2019). Moro-Martínez *et al.* (2023) documented progressive fragmentation of *H. pinnae* DNA in seawater using fragment-size assays, but did not employ real-time PCR, which increases analytical sensitivity. The infectious dose and the gene copy number per parasite remain unknown, so samples negative by conventional PCR but positive by real-time PCR may still contain viable parasites.

A key limitation of this study is the small sample size, as only a single infected individual was available, restricting the generality of the results. Nonetheless, the persistence patterns observed are consistent with established principles of aquatic DNA degradation, supporting their value as a baseline. Future studies should include more individuals and varied environmental conditions to refine persistence estimates and improve the applicability of eDNA monitoring for *H. pinnae* in the field.

In conclusion, this study demonstrates that *H. pinnae* DNA can be detected in seawater for a limited period after host removal, with detectability influenced by amplicon size. The results show both the persistence and gradual degradation of parasite DNA, highlighting the potential and the limits of eDNA approaches for monitoring. Our findings support the use of non-invasive molecular methods to identify *H. pinnae*-free areas and to inform proactive conservation strategies for *P. nobilis* across the Mediterranean.

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Supplementary data

The following supplementary material is available for this article

Detailed description of the electrophoresis method used for the visualisation of PCR products.

Fig. S1: Results of PCR with HAP-F1/HAP-R2 primers from samples obtained with GF filters.

Fig. S2: Results of PCR with HPNF3/HPNR3 primers from samples obtained with GF filters.

Fig. S3: Results of PCR with HAP-F1/HAP-R2 primers from samples obtained with MCE filters in the live-infected (first) experiment.

Fig. S4: Results of PCR with HPNF3/HPNR3 primers from samples obtained with MCE filters in the live-infected (first) experiment.

Fig. S5: Results of PCR with HAP-F1/HAP-R2 primers from samples obtained in the post-mortem (second) experiment.

Fig. S6: Results of PCR with HPNF3/HPNR3 primers from samples obtained in the post-mortem (second) experiment.