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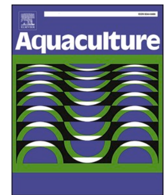

Assessment of the environmental distribution of the protozoan parasite *Perkinsus olseni* by next-generation sequencing, qPCR and histopathology allows the identification of alternative bivalve hosts

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A B S T R A C T

Perkinsus olseni is a protozoan parasite that infects a wide variety of molluscs worldwide, causing economic losses in the aquaculture sector. Consequently, Perkinsosis has been catalogued by the World Organization for Animal Health (O.I.E.) as a notifiable disease, and international measures have been established to control it (O.I.E., 2019). In the present study, we analysed the spatial and temporal distribution of transmissible stages of *Perkinsus olseni* in an endemic area of the parasite from 2016 to 2018. The pathogen was detected using high-throughput sequencing of the 18S rRNA gene and a specific real-time PCR assay (qPCR) in samples of water, sediment and several bivalve species. Histopathological assays were also conducted on bivalve samples.

P. olseni was not detected in environmental samples by qPCR; however, eDNA sequencing revealed its presence in both the water and sediment at all sampled points, showing a seasonal pathogen prevalence. As expected, the parasite was detected in clams, but a few cases were also found in mussels and cockles. The presence of the parasite was confirmed in *Cerastoderma edule* by histology and qPCR using RNA to evaluate the presence of proliferative life stages of the parasite. Therefore, this is the first time that *P. olseni* has been found in *C. edule* but with low abundance and infection intensity levels.

1. Introduction

Many protozoan parasites cause severe diseases and impact host population dynamics and the entire ecosystem (Bråte et al., 2010). The detection and characterization of these parasites are challenging because many of them often infect at low density levels and develop part of their life cycle in alternative hosts or intracellularly (Bass et al., 2015). Moreover, protistan parasites, especially those that belong to the same genus, are morphologically challenging to differentiate by histopathology, even being highly divergent at the molecular level. For this reason, molecular biology-based methods are a good option for accurate characterization of protists and other organisms at the genus or species level (Bass et al., 2015).

The real-time PCR assay (qPCR) is suitable to detect known pathogens using specific primers that target only these species and discriminate among congeneric pathogens. However, the limit of detection should be determined, especially in mixed communities of environmental samples. There are some limitations in this technology, such as the presence of PCR inhibitors (humic acids or heavy metals) in environmental samples and the need to design specific primers in a known genomic region (Porter and Hajibabaei, 2018).

The 18S rRNA metabarcoding technology allows the study of protist diversity in the marine environment, taxonomic identification and the discovery of unknown lineages (Amaral-Zettler et al., 2009; Bråte et al., 2010; de Vargas et al., 2015) using environmental DNA (eDNA) previously amplified by PCR and sequenced on a high-throughput platform

Abbreviations: qPCR, quantitative PCR; eDNA, environmental DNA; O.I.E., World Organization for Animal Health; OTU, Operational Taxonomic Unit; ITS, Intergenic Spacers.

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(Deiner et al., 2017). The analysis of eDNA provides information about parasite (and other organisms) diversity and their life cycles (Bass et al., 2015) by studying different environmental compartments where the parasite might be present (sediment, planktonic size water). The analysis of eDNA is also helpful for the description of alternative or new hosts and the transmission vectors and routes of infection, as in the case of the marine copepod *Paracartia* sp. as the transmission vector of the protozoan parasite *Marteilia refringens* (Boyer et al., 2013; Bass et al., 2015).

Perkinsus olseni is a protist endoparasite classified in the eukaryotic lineage Alveolata, phylum Perkinsozoa and class Perkinsea. The endoparasite infects a wide variety of hosts worldwide and causes perkinsosis disease. In Europe, it was detected on the Atlantic and Mediterranean coasts in *Ruditapes decussatus, R. philippinarum* and *Venerupis pullastra* clams (DaRos and Canzonier, 1985; Azevedo, 1989; Ceschia et al., 1991; Casas et al., 2002; Abollo et al., 2006; Balseiro et al., 2010; Arzul et al., 2012). High mortalities and, therefore, significant economic losses have been associated with this infection across the world (Azevedo, 1989; Sagristà et al., 1996; Villalba et al., 2005; Pretto et al., 2014; Ruano et al., 2015). Particularly in Europe, the infection of *P. olseni* generated a high impact, causing mass mortalities of *R. decussatus*, *R. philippinarum* or *V. pullastra* in Italy (Pretto et al., 2014), southern Portugal (Azevedo, 1989) and Spain (Sagristà et al., 1996; Villalba et al., 2005). As a consequence, *P. olseni* has been catalogued by the World Organization for Animal Health (O.I.E.) as a notifiable parasite (O.I.E, 2019).

Transmission of *Perkinsosis* occurs directly from host to host, indicating a direct lifecycle with three transmissible infective life stages (trophozoite, hypnospore, and zoospore stages). *Perkinsus olseni* has been detected inside the host or as free-living stages allowing a fast horizontal spread of the disease. However, it is unknown which stage is most effective for transmitting the disease in the field (Chu, 1996). Mature trophozoites are spherical-ovoid cells from 13 to 16 μm with a great vacuole taking up the majority of the cytoplasm and a peripheral nucleus (Lester and Davis, 1981; Perkins, 1996; Villalba et al., 2004). These cells are located mainly in the connective tissue of the host. Palintomy proliferation occurs from each mature trophozoite within the host, and immature trophozoites are released from cells that contain 8 to 32 daughter cells. Immature trophozoites enlarge and acquire the vacuole and the typical ring appearance of a mature trophozoite (Perkins, 1996; Villalba et al., 2004). Trophozoites are released to the environment through the faeces or after tissue decomposition from the death hosts. Under unfavourable conditions, these stages enlarge, becoming resistant forms named hypnospores, with diameters of 56–94 μm (Lester and Davis, 1981; Bushek et al., 2002; Villalba et al., 2004; Casas and La-Peyre, 2013). Hypnospores are generally associated with the death of hosts, and their appearance in live animals is limited (Lester and Davis, 1981; Park et al., 2010). Under favourable environmental conditions, the hypnospore matures to become a zoosporangium that contains hundreds of biflagellate zoospores with a size between 3 and 5 μm (Casas et al., 2002) that are released into the environment by the discharge tube to infect another host (Villalba et al., 2004). However, *P. olseni* hypnospore and zoospore stages have only been observed under laboratory conditions and have yet to be detected in the field.

Epizootiological knowledge on *Perkinsus* showed that there is an annual pattern of perkinsosis dynamics influenced by temperature and salinity. On the basis of in vitro analysis, Casas et al. (2002) suggested that *P. olseni* zoosporulation could occur when water temperatures are similar to the temperatures registered in the field from spring to autumn.

The main and novel approach of this study was the combination of several techniques to study the distribution of *P. olseni* in clams (primary host) and other bivalves (*Mytilus galloprovincialis* and *Cerastoderma edule)* and in the environment (sediment and planktonic fractions). DNA metabarcoding based on the 18S ribosomal RNA gene was complemented with a specific qPCR for *P. olseni* previously developed by Ríos et al. (2020). Histopathology analysis and qPCR assays using RNA were also used to describe the proliferative status of the parasite.

2. Materials and methods

2.1. Sampling

Seven consecutive samplings were conducted in Meira (Ría de Vigo, NW Spain), an area of clam production, over three years from 2016 to 2018 (Fig. 1). Temperature and salinity data associated with each sampling time were obtained from the Instituto Tecnolóxico para o Control do Medio Mariño de Galicia database (Intecmar Xunta de Galicia) (INTECMAR, 2010).

In each sampling, 15 mussels (*M. galloprovincialis*), 15 clams (*R. philippinarum*) and 15 cockles (*C. edule*) were collected. Superficial sediment from three different areas of the beach was also taken, mixed and kept at −20 °C until DNA isolation. Seawater was filtered by filters with different pore sizes to obtain 3 fractions of the water column. Seventy-five cubic meters microliters of surface seawater (0–1 m depth) was filtered with a 200-μm plankton net (mesoplankton, >200 μm). Then, a volume of 40 L was filtered through a 65 μm pore size net to obtain the microplankton (200–65 μm), and finally, 2 L of this water was again filtered through a 0.22 μm pore size to retain the nanoplankton and picoplankton fraction (65–0.22 μm) (Fig. 1B). All filters were kept at −20 °C until molecular analysis (18S rRNA sequencing and qPCR).

Bivalves were carefully extracted from the shell, and half of the body was fixed in Davidson solution (Shaw and Battle, 1957) for 24 h for histological analysis. The other half of the animal was used for DNA and RNA isolation. A small fragment of the gill, gonad, mantle and digestive gland was pooled in a single sample from each individual and preserved in ethanol (99–100%) for isolating DNA and preserved in RNA-Later (Merck, Darmstadt, Germany) for isolating the RNA.

2.2. DNA and RNA isolation

DNAs from a total of 24 samples of sediment and water column fractions were isolated using the PowerSoil DNA isolation Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Total DNA and RNA from a total of 20 samples of molluscs were isolated using the Maxwell 16 Blood Purification DNA Kit (Promega, Madison, USA) and Maxwell 16 LEV Simply RNA Tissue Kit (Promega, Madison, USA), respectively, according to the manufacturer's instructions. DNA and RNA quality and quantity were estimated with a NanoDrop™ 1000 spectrophotometer (NanoDrop Technologies, Inc., DE, USA). cDNA synthesis was performed from RNA samples following the NZY First-Strand cDNA Synthesis Kit (Nzytech, Lisbon, Portugal) protocol.

2.3. 18S SSU rRNA sequencing by Illumina

DNA from bivalves collected at each sampling point ($N = 15$) was pooled. The V9 region of the 18S SSU rRNA gene was amplified using DNA isolated from environmental compartments and from pooled bivalves using the universal eukaryotic-specific primers 1380F/1510R from Amaral-Zettler et al. (2009). The amplicons obtained (180 bp long) were purified using the Qiaquick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol and quantified with the NanoDrop™. A library was prepared using the Herculase II Fusion DNA Polymerase Nextera XT Index Kit V2 (Illumina), and paired-end sequencing (2 × 300) was performed on an Illumina MiSeq platform (Macrogen, Seoul, Shouth Korea).

Bioinformatic analysis was detailed in Ríos-Castro et al. (2021). Briefly, a custom reference database using sequences of the 18S gene from eukaryotes was designed to assign an operational taxonomic unit (OTU) to the sequences obtained after Illumina sequencing. Data analysis was performed using the Microbial Genomics Module package of CLC Workbench 12 software (Qiagen, Hilden, Germany). Raw sequences were trimmed, and the reference database OTU clustering procedure was performed. Then, sequences identified as being from *P. olseni* were extracted from the OTU table after the clustering procedure for each of

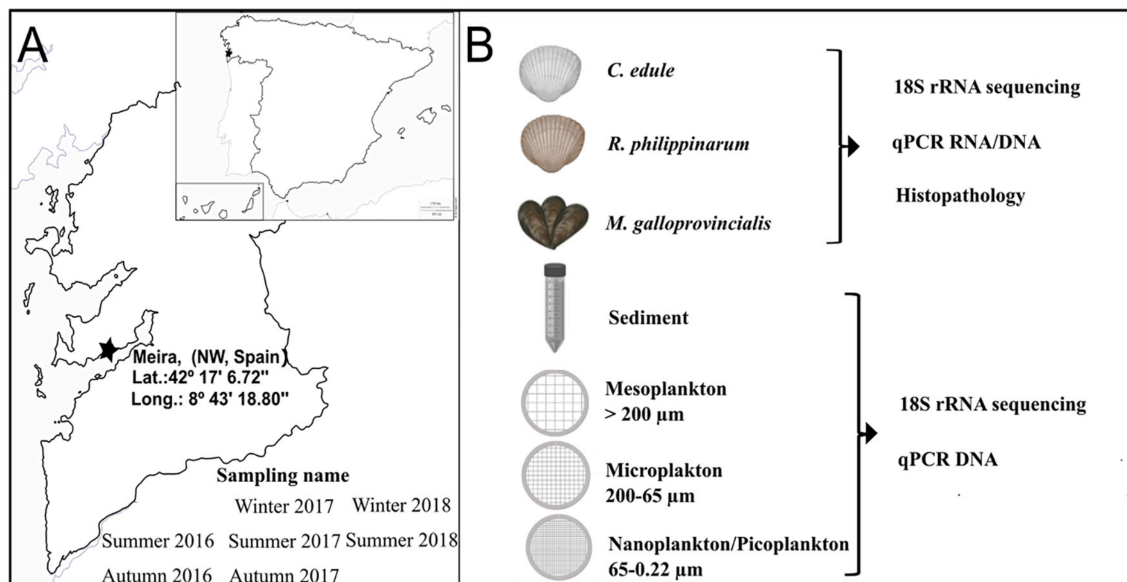

Fig. 1. A) Sampling performed in Meira (Ría de Vigo, NW, Spain) from 2016 to 2018. B) Diagnosis techniques performed for each bivalve and environmental compartment sampled.

the samples, and they were managed individually. The number of *Perkinsus* reads in each sample corresponded to abundance values from bivalves and environmental compartments.

2.4. Specific real time PCR

A specific real-time PCR (qPCR) assay was performed using DNA and cDNA extracted from individual samples of bivalves and DNA of environmental samples. The detection of *P. olseni* was conducted with specific primers (Perk-ITS-q F1 and Perk-ITS-q R2) that amplify the intergenic spacer regions 1 and 2 (ITS-1 and ITS-2) of the ribosomal RNA following the criteria described by Ríos et al. (2020). The intensity of the infection in clams was calculated following the Pfaffl method (Pfaffl, 2001) by normalizing the threshold cycle (Ct) values using the 18S gene as a reference gene (Moreira et al., 2012). To confirm positive results, PCR amplicons were purified using the manufactured protocol kit Illustra ™ ExoProStar 1-Step (Merck, Darmstadt, Germany) and sequenced (Eurofins Genomics, Ebersberg, Germany).

2.5. Histopathology

Transversal sections of fixed bivalves were prepared to include all the different organs of the animals. Samples were automatically processed (Leica TP 1020, Leica Microsystems, Wetzlar, Germany), embedded in paraffin blocks and cut into 5-μm thick sections on a microtome (Leica RM2255; Leica Microsystems, Wetzlar, Germany). Sections were deparaffinized, rehydrated and stained with haematoxylin-eosin. Tissues were examined under an optical microscope (Nikon Eclipse 80i, Nikon, Tokyo, Japan) at 40× to evaluate the prevalence of *Perkinsus* spp. and the presence of histopathological alterations.

3. Results

3.1. Presence of Perkinsus olseni in marine environmental compartments

A total of 24 environmental samples, including sediment and planktonic fractions, were analysed by sequencing the 18S rRNA gene. After recovering the raw reads, trimming and OTU clustering procedures, specific reads for *Perkinsus* were selected. In parallel, a qPCR assay for the specific detection of *P. olseni* was also conducted in these environmental samples.

Perkinsus was not detected in the marine environment compartments by specific qPCR; however, it was possible to detect the parasite by sequencing the 18S rRNA gene in all the samplings of marine environmental fractions but, in general, with very low absolute abundance (Fig. 2A). Environmental factors such as temperature and salinity did not show abnormal variations throughout the studied period, and no clear temporal distribution of the parasite could be established. *Perkinsus* presented a maximum prevalence in autumn 2016, when the abundance values were higher in all the environmental compartments (Fig. 2).

The parasite was found in the sediment in all the samplings (Fig. 2B), with the highest abundances in autumn 2016 and 2017. In the mesoplankton, *P. olseni* was only detected at very low levels in winter and summer 2017 and in summer 2018 (Fig. 2C). The highest abundance in the environment was detected in the microplankton in autumn 2016, although the parasite was not present in all samplings (Fig. 2D). In the nanoplankton/picoplankton, the parasite was always detected (with the exception of autumn 2017, when no data could be obtained) (Fig. 2E), with the highest abundance level also in autumn 2016.

Although it is not possible to establish clear temporal and spatial variation of the presence of the pathogen in the different environmental fractions, autumn seemed to be the time of the year with the highest prevalence in the environment over the studied period.

3.2. Presence of Perkinsus olseni in bivalves

Twenty samples of pooled DNA extracted from bivalve tissues were taken for massive sequencing of the 18S rRNA gene. In parallel, histology and qPCR assays for the specific detection of *P. olseni* were also performed in individual animals.

By massive sequencing, *P. olseni* was mainly detected in clams at all sampling points. The highest abundance level was obtained from summer 2016 to winter 2017, with a maximum detection in autumn 2016. The same pattern was observed from summer 2017 to winter 2018, although with lower abundances. In general, a decrease in the presence of the parasite could be observed over the years (Fig. 3A).

Histological examination of tissue sections of clams revealed the presence of the parasite and pathological alterations in clams. The highest prevalence was observed in summer 2016 (40%), followed by winter 2017 (32%) and winter 2018 (20%). Low incidence levels were

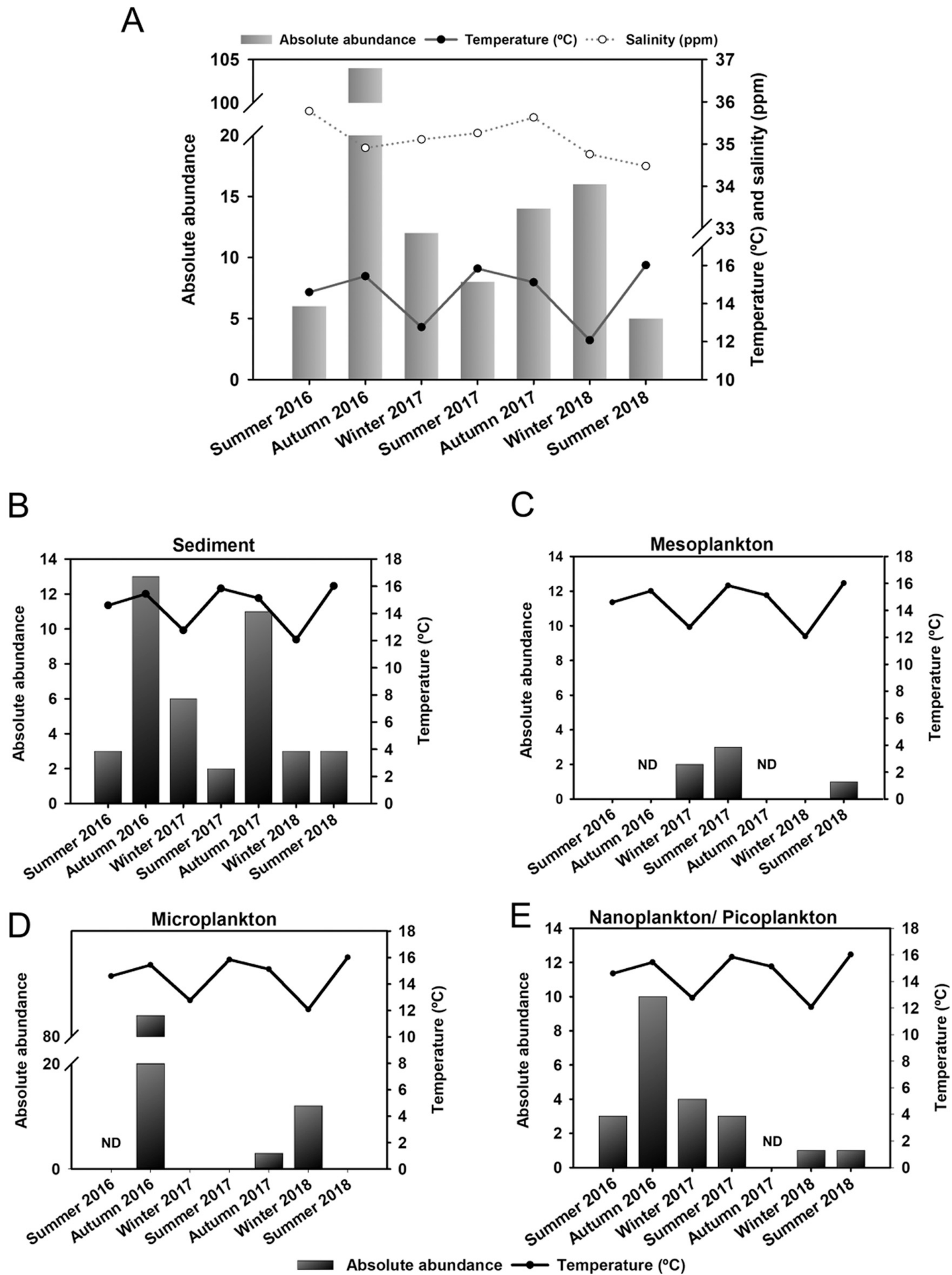

Fig. 2. *P. olseni* abundance obtained by 18S SSU ribosomal RNA V9 region from: A) The combination of all environmental sampling, B) Sediment, C) mesoplankton (>200 μm), D) microplankton (200–65 μm), E) nanoplankton-picoplankton (65–0.22 μm). The average temperature and salinity from the same month of each sampling episode were represented from Intecmar data.

obtained in autumn 2016 and autumn 2017 (6% in both cases). *Perkinsus* was not detected in summer 2017 or summer 2018 (Fig. 3B).

A similar pattern of prevalence was observed by qPCR. A general increase in the incidence was observed from summer to winter in all sampling periods, with the highest levels detected in winter every year. The prevalence was higher in 2017 than in 2018, showing a general decrease in occurrence from 2016 to 2018 (Fig. 4A). Moreover, the infection level of the parasite remained constant in all years and seasons without significant differences (Fig. 4B).

To detect the active proliferation of the pathogen (replicative stage) inside the bivalves, we used RNA extracted from the same bivalves and conducted new qPCR assays. When clam cDNA samples were used, an

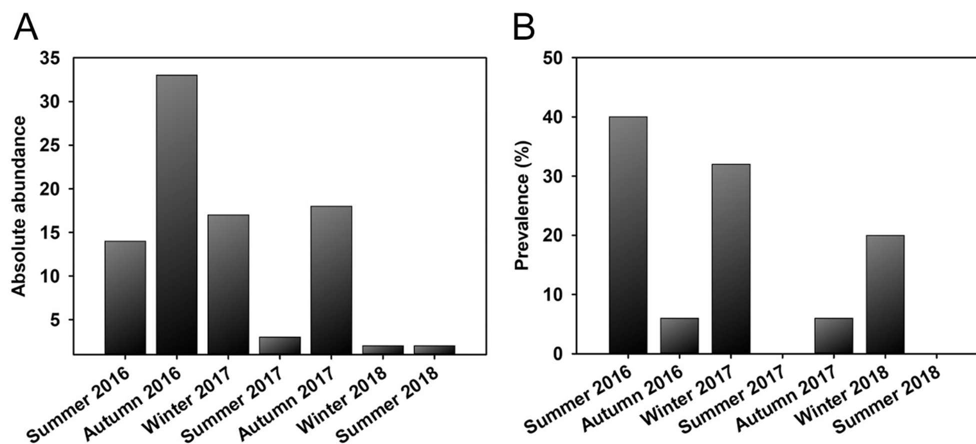

Fig. 3. A) Absolute abundance of *P. olseni* obtained by 18S SSU rRNA V9 region sequencing in the clam *R. philippinarum* from each sampling. B) Prevalence of *P. olseni* in the clam *R. philippinarum* by histopathological assay.

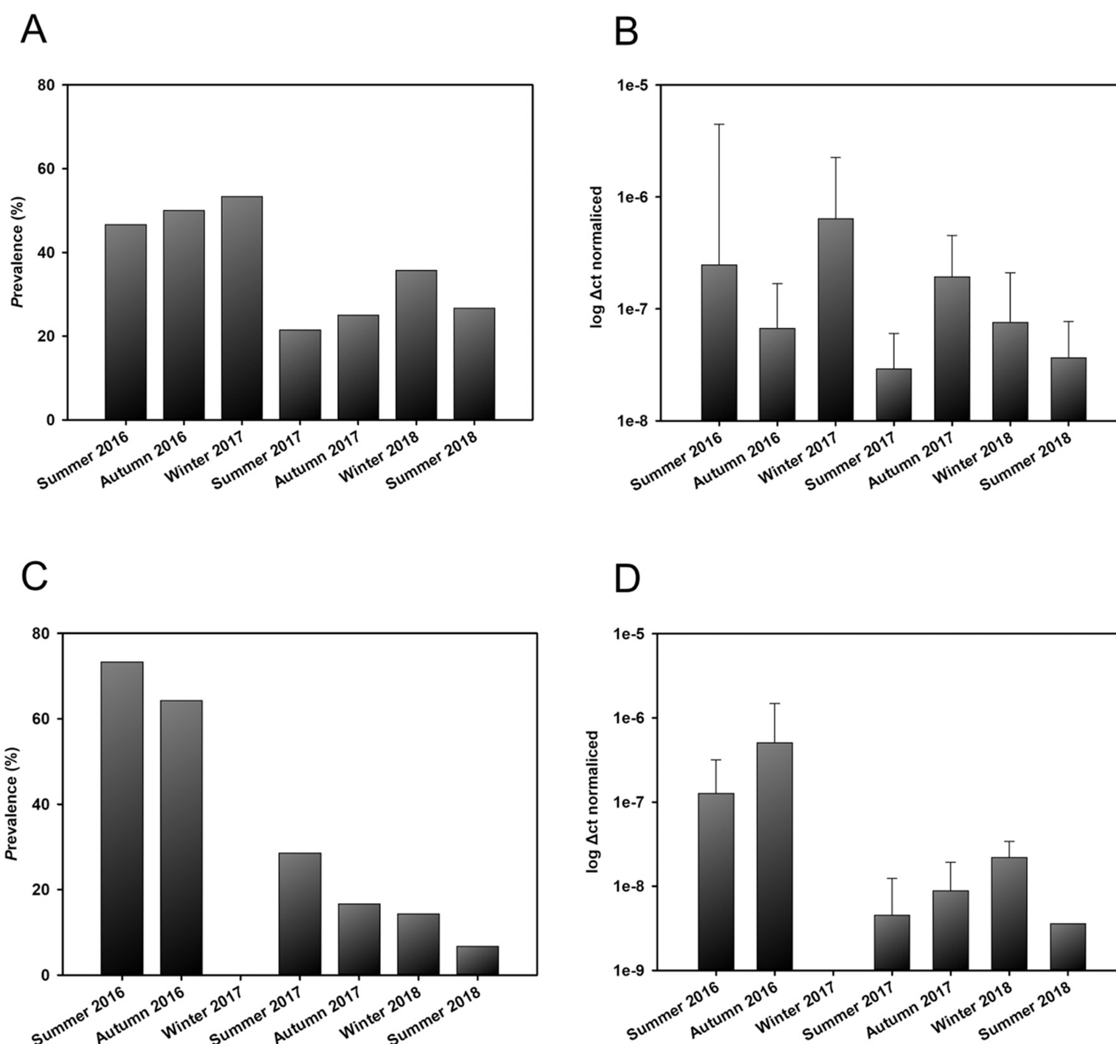

Fig. 4. A) Prevalence of *P. olseni* in the clam *R. philippinarum* by qPCR using DNA extracted from tissue. B) *P. olseni* infection intensity (log ΔCt normalized) from *R. philippinarum* tissue DNA. C) *P. olseni* prevalence in the clam *R. philippinarum* by qPCR using cDNA extracted from tissue D) *P. olseni* infection intensity (log ΔCt normalized) from *R. philippinarum* tissue cDNA.

opposite pattern of prevalence among seasons was observed compared with the previous results obtained using DNA (Fig. 4C). In general, higher prevalence values were obtained in summer, followed by lower prevalence in autumn and winter. In summer 2018, only 6% prevalence was observed, compared with 78% prevalence in summer 2016 and 30% prevalence in summer 2017. Thus, the lower prevalence also indicates a

general decrease in the proliferative stage of the parasite from 2016 to 2018, showing a downward trend over the years again. The infection level of the parasite also remained constant in all years and seasons, with no significant differences observed (Fig. 4D).

eDNA analysis also allowed the description of an alternative host for this pathogen. Our study included mussels and cockles that a priori are not affected by the parasite. By massive sequencing, *Perkinsus* was detected in both bivalve species at very low abundance. The parasite was detected in mussels (one read) in winter 2017 and autumn 2017 and in cockles in summer 2017 (one read) (data not shown).

The parasite was never observed by histology in mussel tissues (data not shown). However, *P. olseni* was detected in cockle tissues in the histological study in one individual in the summer 2017 sampling (6.6% prevalence) and in 2 specimens from the winter 2018 sampling (22.2% prevalence). The infection intensity was low (1–5 trophozoites per section), and haemocyte infiltration that could be associated with *Perkinsus* presence was frequently observed. As in clams, mature trophozoites were located isolated in the connective tissue near the digestive gland and gonad. They were spherical and ovoid (7.82 ± 1.01 SE length and 7.28 ± 0.75 SE width). The typical "single ring" appearance was

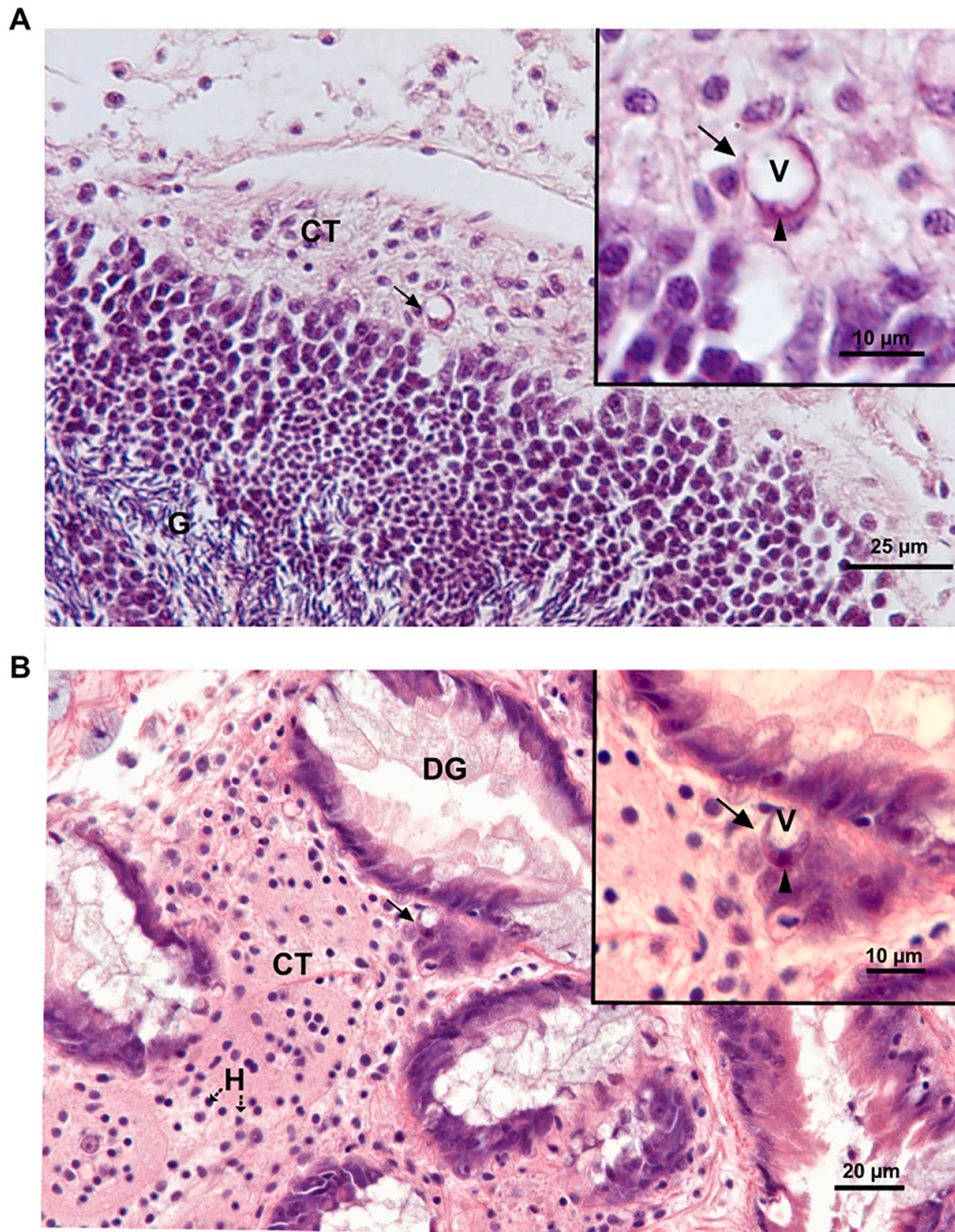

Fig. 5. Transversal tissue sections of the cockle *C. edule.* A) *P. olseni* mature trophozoite (arrow) located on the connective tissue and near the gonad. Bar scale: 25 µm and 10 µm. B) *P. olseni* mature trophozoite (arrow) located on the connective tissue near of the digestive gland. A high number of haemocytes were observed in the zone of the connective tissue indicating tissue infiltration. Bar scale: 25 µm and 10 µm. In both amplified images (100×) the "single ring" appearance was observed in trophozoites, with a big vacuole that occupies the majority of the cytoplasm and a peripheral nucleus (arrowhead). CT: connective tissue; DG: digestive gland; V: vacuole; G: gonad; H: haemocytes.

observed in trophozoites, with a great vacuole that occupies the majority of the cytoplasm and a peripheral nucleus enclosing an evident nucleolus (Fig. 5).

By qPCR, *P. olseni* was only detected in two pools of DNA extracted from mussels in winter and autumn 2017. In cockles, *P. olseni* was also detected in only one pool of DNA in summer 2017 (data not shown).

When cDNA was used for qPCR assays (replicative stage of the parasite), the parasite was never detected in mussels. In contrast, two samples of tissues extracted from cockles were positive. Interestingly, the positive samples from winter 2018 were also positive by histology.

All the positive PCR products were sequenced to confirm the identity of the amplified fragments, and all of them showed 99–100% identity with *P. olseni* (LC431768.1) (data not shown).

4. Discussion

The surveillance of parasites affecting cultured fish and shellfish is conducted mainly in the affected hosts. However, the detection of these potential pathogens in the marine environment is a strenuous activity. Although *Perkinsus olseni* is a widely studied pathogen, most of the studies are focused on the interactions between hosts and pathogens, explaining how the progression of the infection occurs (Sagristà et al., 1995; Ordás et al., 2001; Casas, 2002; Chintala et al., 2002), describing the immune responses after the infection (Anderson, 1999a; Anderson, 1999b; Ordás et al., 2000; Gauthier and Vasta, 2002), the pathogen prevalence in host tissues after environmental changes such as temperature and salinity (Goggin et al., 1990; Ragone and Burreson, 1993; Ragone and Burreson, 1994; Gauthier and Vasta, 1995; Casas, 2002) and the geographical dissemination of *Perkinsus* species (Ek-Huchim et al., 2017).

However, infected bivalves release parasites through faeces or decayed tissue from dead organisms to the environment, and as a consequence, *P. olseni* life stages are free-living in the field until they infect new hosts (Bushek et al., 2002; Park et al., 2010). To date, however, only limited studies have reported the presence of *Perkinsus* spp. in the water column (Ragone-Calvo et al., 2003; Audemard et al., 2006) and sediment (Park et al., 2010) using different techniques, such as real-time PCR assays (Audemard et al., 2006), flow cytometry (Ragone-Calvo et al., 2003) or immunofluorescence assays and Western blotting (Park et al., 2010). The low prevalence of parasites in the environment and other similar species or interfering contaminants could limit detection in environmental samples.

Recently, DNA metabarcoding has been used more often in ecological studies to monitor eukaryotic and prokaryotic organisms in the environment (Maritz et al., 2017; Polinski et al., 2019; Sunagawa et al., 2020). The main benefit of this assay is that it is possible to obtain in the same analysis all the eukaryotic diversity present in a sample by amplifying and sequencing only a particular conserved gene, which makes this technique a non-invasive, less time-consuming and more adequate method without the need to detect specific organisms.

The protist parasite *P. olseni* was detected in eDNA and in clams throughout the three years of our study by sequencing. This study was the first where transmissible infective stages could be associated with environmental compartments in almost all sampling periods. Based on the known parasite sizes of each transmissible life stage and according to the sequencing data obtained in this study, the different life stages of the parasite could be associated with different compartments in the ecosystem (Fig. 6). Clam mortality increases after spring and mid-summer peak prevalence (Villalba et al., 2005). In this period, the enlargement of trophozoites occurs in moribund hosts, and they are released after clam death or by faeces elimination (Bushek et al., 2002; Park et al., 2010). Resistance forms known as hypnospores are released to the environment (Ray, 1954; Perkins and Menzel, 1967; Casas et al., 2002; Villalba et al., 2004). In sediment, we detected the pathogen throughout the years, with the highest abundance levels detected in autumn 2016 and autumn 2017, when host mortalities were more likely to occur (Villalba et al., 2005). Interestingly, a similar seasonal abundance pattern was obtained in clams by sequencing. Trophozoites and

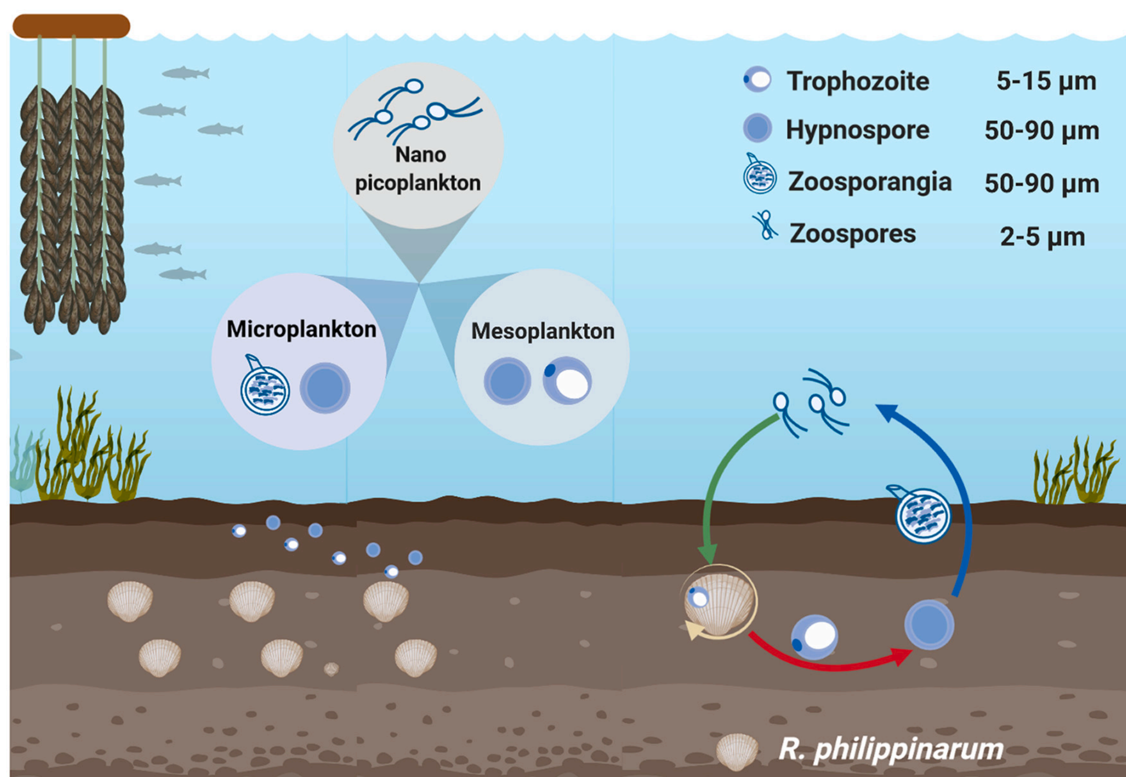

Fig. 6. *P. olseni* spatial distribution in environmental compartments, according to the life cycle of the parasite and sequencing results obtained from the environment and bivalves.

hypnospores are the life stages detected in infected hosts, and they are released to the sediment after clam death or by faeces, suggesting the presence of trophozoites and hypnospores mainly in sediment. The highest abundance levels of *P. olseni* were detected in the microplankton size in autumn 2016. The diameter range of *P. olseni* hypnospores is 56–94 μm (Lester and Davis, 1981); therefore, hypnospores could be present in this compartment, and with favourable environmental conditions, the zoosporulation process occurs, and the hypnospores turn into zoosporangia (50–90 μm), which are also present in this compartment. Hundreds of biflagellate zoospores (5–15 μm) are released to the environment by zoosporangia, and consequently, the parasite is propagated to another host by gill filtration (Villalba et al., 2004). In the nanoplankton/picoplankton fraction (65–0.22 μm), the highest *P. olseni* abundance was from autumn 2016, and mainly zoospores could be detected. *Perkinsus olseni* could also be present in the mesoplankton (>200 μm), although with the lowest prevalence. Ocean currents could remove the sediment, and *P. olseni* could be released and retained on larger planktonic components, also explaining their presence in this compartment.

Since clams are a well-known susceptible host of *P. olseni*, it was possible to obtain a clear seasonal pattern of the prevalence of *P. olseni* in the proliferative stage and nonproliferative stage by qPCR assay. Opposite patterns of *P. olseni* prevalence were observed by real-time PCR when DNA and cDNA samples were analysed in clams. These differences could be explained by the proliferative status of the parasite, which would be represented in cDNA samples, whereas the accumulation of parasites in clam tissue from previous proliferation periods could be detected in DNA samples (Not et al., 2009; Bass et al., 2015). In summer periods, more potentially proliferative *P. olseni* (cDNA) was observed than in the non-proliferative stage (DNA). In autumn, the prevalence decreased in cDNA samples, while an increase was observed in DNA samples, suggesting that the proliferation of the parasite continues in autumn but at lower levels than in summer, and the accumulation of the parasite in host tissue occurs from the last replication stage. In winter, the proliferation of the parasite decreased, and the lowest prevalence values were detected in cDNA samples. However, the accumulation of parasite cells that are not in proliferative stages may explain the higher prevalence.

Moreover, when the water temperature is above 15 °C (summer-early autumn), higher infection levels and prevalence of *P. olseni* are known to be detected than when the water temperature is below 15 °C (winter), showing a seasonal pattern of perkinsosis disease (Azevedo et al., 1990; Villalba et al., 2005). The average water temperature in Meira was 15–16 °C in summer-autumn during the three years sampled (Intecmar data) and 12–13 °C in winter, so our prevalence data from cDNA samples are related to seawater temperature, being concordant in a seasonal pattern of perkinsosis prevalence. Although a seasonal pattern of prevalence according to seawater temperature has been observed in clams, the infection intensity was low and constant in sampling, and no significant differences in the intensity infection level were detected throughout the period studied.

When these qPCR results were compared with the metabarcoding from sequencing the DNA amplicon of the V9 18S ribosomal RNA region in bivalves, the highest abundance of the parasite in clams was also detected in autumn in all the years sampled. As previously described, there was a downward trend in pathogen prevalence during our study period. Therefore, we could assume that currently, *P. olseni* in environmental waters in Ría de Vigo does not pose an imminent risk to the culture of bivalve molluscs; nevertheless, it is important to maintain the monitoring of the disease. The use of high-throughput sequencing from environmental DNA samples could be helpful to establish an early monitoring predictive model of the disease over the years, considering other environmental factors and evaluating the long-term effect of climate change on the parasite life cycle.

P. olseni was detected with very low infection intensity from DNA samples of the mussel *M. galloprovincialis* using qPCR and sequencing of the V9 region of the 18S rRNA gene. However, *P. olseni* was not observed in cDNA samples by qPCR or by histopathological analysis. Mussels filtered between 2 and 8 L/h of water (Wijsman and Smaal, 2017), and *P. olseni* could be filtered by mussels and detected without proliferation taking place, the possible reason why *P. olseni* was detected only in individual DNA samples with very low abundance, proving the high sensitivity levels of the 18S metabarcoding and the specific qPCR assay used.

However, active infection of *Perkinsus olseni* was detected in cockles with very low abundance by high-throughput sequencing of the V9 region of the 18S rRNA gene, suggesting the high sensitivity of this technique to detect very low levels of the parasite in the environment. This new finding was corroborated with the specific qPCR of cDNA, confirming the cockle *C. edule* as a new host of the parasite *P. olseni*, which has the capacity to proliferate in this bivalve. In this case, the parasite was also detected by histopathology, although no associated lesions were detected. In previous studies, *C. edule* has been reported to be infected by *Perkinsus chesapeaki* on the Mediterranean coast (Carrasco et al., 2014), and *P. olseni* has also been detected in other *Cerastoderma* species (*C. glaucum*) (Ramilo et al., 2015), but this is the first report of *P. olseni* isolated from the cockle *C. edule*. However, only a few isolated trophozoites were detected in the infected animals, causing no tissue damage. The role of *C. edule* as a new host of the pathogen has to be studied deeply.

P. olseni was only detected by high-throughput sequencing of the V9 region of the 18S rRNA gene in sediment and different planktonic fractions, even at very low abundance, which makes high-throughput sequencing an appropriate technique for environmental samples. Environmental conditions such as nutrient availability, temperature and salinity conditions play an essential role in the prezoosporulation and zoosporulation of *Perkinsus olseni* (Goggin et al., 1990; Casas et al., 2002).

In this study, we were not able to establish a precise temporal distribution of the different life stages of the parasite in the environment. Nevertheless, we have demonstrated that replication of the parasite is a continuous process that occurs throughout the year, as RNA of the parasite was found by qPCR from almost all samplings.

This study led us to conclude that the high-throughput sequencing of the 18S rRNA gene and a specific real-time PCR assay (qPCR) combined with classical methods such as histology allowed us to determine the prevalence, intensity and proliferative status of *Perkinsus olseni* in several bivalve species and environmental compartments. This approach could be used to improve the management of the disease and even the prevention of mass mortality episodes.

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Declaration of Competing Interest

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

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References

Abollo, E., Casas, S.M., Ceschia, G., Villalba, A., 2006. Differential diagnosis of *Perkinsus* species by polymerase chain reaction-restriction fragment length polymorphism assay. Mol. Cell. Probes 20 (6), 323–329. https://doi.org/10.1016/j.mcp.2006.04.001.

Amaral-Zettler, L.A., McCliment, E.A., Ducklow, H.W., Huse, S.M., 2009. A method for studying protistan diversity using massively parallel sequencing of V9 hypervariable regions of small-subunit ribosomal RNA genes. PLoS One 4 (7), e6372. https://doi.org/10.1371/journal.pone.0006372 (Erratum in: PLoS One 4(12). doi: 10.1371/annotation/50c43133-0df5-4b8b-8975-8cc37d4f2f26).

Anderson, R.S., 1999a. *Perkinsus marinus* secretory products modulate superoxide anion production by oyster (*Crassostrea virginica*) haemocytes. Fish Shellfish Immunol. 9, 51–60. https://doi.org/10.1006/fsim.1998.0174.

Anderson, R.S., 1999b. Lack of hemocyte chemiluminescence stimulation by *Perkinsus marinus* in eastern oysters *Crassostrea virginica* with Dermo disease. J. Aquat. Anim. Health 11, 179–182. https://doi.org/10.1577/1548-8667(1999)011<0179:LOHCSB>2.0.CO;2.

Arzul, I., Chollet, B., Michel, J., Robert, M., Garcia, C., Joly, J.P., Miossec, L., 2012. One *Perkinsus* species may hide another: characterization of *Perkinsus* species present in clam production areas of France. Parasitology 139 (13), 1757–1771. https://doi.org/10.1017/S0031182012001047.

Audemard, C., Ragone-Calvo, L.M., Paynter, K.T., Reece, K.S., Burreson, E.M., 2006. Real-time PCR investigation of parasite ecology: in situ determination of oyster parasite *Perkinsus marinus* transmission dynamics in lower Chesapeake Bay. Parasitology 132 (6), 827–842. https://doi.org/10.1017/S0031182006009851.

Azevedo, C., 1989. Fine structure of *Perkinsus atlanticus* n. sp. (Apicomplexa, Perkinsea) parasite of the clam *Ruditapes decussatus* from Portugal. J. Parasitol. 75 (4), 627–635.

Azevedo, C., Corral, L., Cachola, R., 1990. Fine structure of zoosporulation in *Perkinsus atlanticus* (Apicomplexa: Perkinsea). Parasitology 100 (3), 351–358. https://doi.org/10.1017/s0031182000078616.

Balseiro, P., Montes, J., Fernández-Conchas, R., Novoa, B., Figueras, A., 2010. Comparison of diagnostic techniques to detect the clam pathogen *Perkinsus olseni*. Dis. Aquat. Org. 90 (2), 143–151. https://doi.org/10.3354/dao02194.

Bass, D., Stentiford, G.D., Littlewood, D.T.J., Hartikainen, H., 2015. Diverse applications of environmental DNA methods in parasitology. Trends Parasitol. 31 (10), 499–513. https://doi.org/10.1016/j.pt.2015.06.013.

Boyer, S., Chollet, B., Bonnet, D., Arzul, I., 2013. New evidence for the involvement of *Paracartia grani* (Copepoda, Calanoida) in the life cycle of *Marteilia refringens* (Paramyxea). Int. J. Parasitol. 43 (14), 1089–1099. https://doi.org/10.1016/j.ijpara.2013.07.008.

Bråte, J., Logares, R., Berney, C., Ree, D.K., Klaveness, D., Jakobsen, K.S., Shalchian-Tabrizi, K., 2010. Freshwater Perkinsea and marine-freshwater colonizations revealed by pyrosequencing and phylogeny of environmental rDNA. ISME J. 4 (9), 1144–1153. https://doi.org/10.1038/ismej.2010.39.

Bushek, D., Ford, S.E., Chintala, M.M., 2002. Comparison of in vitro-cultured and wild-type *Perkinsus marinus*. III. Fecal elimination and its role in transmission. Dis. Aquat. Org. 51 (3), 217–225. https://doi.org/10.3354/dao051217.

Carrasco, N., Rojas, M., Aceituno, P., Andree, K.B., Lacuesta, B., Furones, M.D., 2014. *Perkinsus chesapeaki* observed in a new host, the European common edible cockle *Cerastoderma edule*, in the Spanish Mediterranean coast. J. Invertebr. Pathol. 117, 56–60. https://doi.org/10.1016/j.jip.2014.01.009.

Casas, S.M., 2002. Estudio de la perkinsosis en almeja fina, *Tapes decussatus* (Linnaeus 1758) de Galicia [dissertation thesis]. University of Santiago de Compostela, Santiago de Compostela, Spain.

Casas, S.M., La-Peyre, J.F., 2013. Identifying factors inducing trophozoite differentiation into hypnospores in *Perkinsus* species. Eur. J. Protistol. 49 (2), 201–209. https://doi.org/10.1016/j.ejop.2012.07.004.

Casas, S.M., Villalba, A., Reece, K.S., 2002. Study of perkinsosis in the carpet shell clam *Tapes decussatus* in Galicia (NW Spain). I. Identification of the aetiological agent and in vitro modulation of zoosporulation by temperature and salinity. Dis. Aquat. Org. 50 (1), 51–65. https://doi.org/10.3354/dao050051.

Ceschia, C., Zentilin, A., Giorgetti, G., 1991. Presenza di *Perkinsus* in vongole veraci (*Ruditapes philippinarum*) alleviate nel Nord-Est Italia. Boll. Soc. It. Patol. Ittica 5, 101–108.

Chintala, M.M., Bushek, D., Ford, S.E., 2002. Comparison of in vitro-cultured and wild-type *Perkinsus marinus*. II. Dosing methods and host response. Dis. Aquat. Org. 51 (3), 203–216. https://doi.org/10.3354/dao051203.

Chu, Fu-Lin, 1996. Laboratory investigations of susceptibility, infectivity and transmission of *Perkinsus marinus* in oysters. J. Shellfish Res. 15, 57–66.

DaRos, L., Canzonier, W.J., 1985. *Perkinsus*, a protistan threat to bivalve culture in the Mediterranean basin. Bull. Eur. Ass. Fish. Pathol. 5, 23–25.

de Vargas, C., Audic, S., Henry, N., Decelle, J., Mahé, F., Logares, R., et al., 2015. Ocean plankton. Eukaryotic plankton diversity in the sunlit ocean. Science 348 (6237), 1261605. https://doi.org/10.1126/science.1261605.

Deiner, K., Bik, H.M., Mächler, E., Seymour, M., Lacoursière-Roussel, A., Altermatt, F., de-Vere, N., Pfrender, M.E., Bernatchez, L., 2017. Environmental DNA metabarcoding: transforming how we survey animal and plant communities. Mol. Ecol. 26 (21), 5872–5895. https://doi.org/10.1111/mec.14350.

Ek-Huchim, J.P., Aguirre-Macedo, M.L., Améndola-Pimenta, M., Vidal-Martínez, V.M., Pérez-Vega, J.A., Simá-Alvarez, R., Rodríguez-Canul, R., 2017. Genetic signature analysis of *Perkinsus marinus* in Mexico suggests possible translocation from the Atlantic Ocean to the Pacific coast of Mexico. Parasit. Vectors 10 (1), 372. https://doi.org/10.1186/s13071-017-2304-4.

Gauthier, J., Vasta, G., 1995. In vitro culture of the eastern oyster parasite *Perkinsus marinus*: optimization of the methodology. J. Invertebr. Pathol. 66, 156–168. https://doi.org/10.1006/jipa.1995.1079.

Gauthier, J., Vasta, G., 2002. Effects of plasma from bivalve mollusk species on the in vitro proliferation of the protistan parasite *Perkinsus marinus*. J. Exp. Zool. 292, 221–230. https://doi.org/10.1002/jez.10013.

Goggin, C.L., Sewell, K.B., Lester, R.J.G., 1990. Tolerances of *Perkinsus* spp. (Protozoa, Apicomplexa) to temperature chlorine and salinity. J. Shellfish Res. 9, 1445–1448.

INTECMAR, 2010. Instituto Tecnolóxico para o Control do Medio Mariño de Galicia, Xunta de Galicia. http://www.intecmar.gal/Ctd/Default.aspx (Accesed June 06, 2021).

Lester, R.J.G., Davis, G.H.G., 1981. A new *Perkinsus* species (Apicomplexa, Perkinsea) from the abalone *Haliotis ruber*. J. Invertebr. Pathol. 37, 181–187. https://doi.org/10.1016/0022-2011(81)90073-2.

Maritz, J.M., Rogers, K.H., Rock, T.M., Liu, N., Joseph, S., Land, K.M., Carlton, J.M., 2017. An 18S rRNA workflow for characterizing protists in sewage, with a focus on zoonotic trichomonads. Microb. Ecol. 74 (4), 923–936. https://doi.org/10.1007/s00248-017-0996-9.

Moreira, R., Balseiro, P., Romero, A., Dios, S., Posada, D., Novoa, B., Figueras, A., 2012. Gene expression analysis of clams *Ruditapes philippinarum* and *Ruditapes decussatus* following bacterial infection yields molecular insights into pathogen resistance and immunity. Dev. Comp. Immunol. 36 (1), 140–149. https://doi.org/10.1016/j.dci.2011.06.012.

Not, F., del Campo, J., Balagué, V., de Vargas, C., Massana, R., 2009. New insights into the diversity of marine picoeukaryotes. PLoS One 4 (9), e7143. https://doi.org/10.1371/journal.pone.0007143.

O.I.E, 2019. Manual de las pruebas diagnóstico para los animales acuáticos. Perkinsus olseni. https://www.oie.int/es/que-hacemos/normas/codigos-y-manuales/acceso-en-linea-al-manual-acuatico/?id=169&L=1&htmfile=chapitre_perkinsus_olseni.htm (Accessed June 03 2021).

Ordás, M.C., Ordás, A., Beloso, C., Figueras, A., 2000. Immune parameters in carpet shell clams naturally infected with *Perkinsus atlanticus*. Fish Shellfish Immunol. 10 (7), 597–609. https://doi.org/10.1006/fsim.2000.0274.

Ordás, M.C., Gómez-León, J., Figueras, A., 2001. Histophatology of the infection by *Perkinsus atlanticus* in three clam species (*Ruditapes decussatus*., *R. philippinarum* and *R. pullastra*) from Galicia (NW Spain). J. Shellfish Res. 20, 1019–1024.

Park, K.I., Yang, H.S., Kang, H.S., Cho, M., Park, K.J., Choi, K.S., 2010. Isolation and identification of *Perkinsus olseni* from feces and marine sediment using immunological and molecular techniques. J. Invertebr. Pathol. 105 (3), 261–269. https://doi.org/10.1016/j.jip.2010.07.006.

Perkins, F.O., 1996. The structure of *Perkinsus marinus* (Mackin, Owen and Collier,1950) Levine 1978, with comments on taxonomy and phylogeny of *Perkinsus* spp. J. Shellfish Res. 15 (1), 67–87.

Perkins, F.O., Menzel, R.W., 1967. Ultrastructure of sporulation in the oyster pathogen *Dermocystidium marinum*. J. Invertebr. Pathol. 9 (2), 205–229.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29 (9), e45 https://doi.org/10.1093/nar/29.9.e45.

Polinski, J.M., Bucci, J.P., Gasser, M., Bodnar, A.G., 2019. Metabarcoding assessment of prokaryotic and eukaryotic taxa in sediments from Stellwagen Bank National Marine Sanctuary. Sci. Rep. 9 (1), 14820. https://doi.org/10.1038/s41598-019-51341-3.

Porter, T.M., Hajibabaei, M., 2018. Scaling up: a guide to high-throughput genomic approaches for biodiversity analysis. Mol. Ecol. 27 (2), 313–338. https://doi.org/10.1111/mec.14478.

Pretto, T., Zambon, M., Civettini, M., Caburlotto, G., Boffo, L., Rossetti, E., Arcangeli, G., 2014. Massive mortality in Manila clams, *Ruditapes philippinarum*, farmed in the Lagoon of Venice caused by *Perkinsus olseni*. Bull. Eur. Assoc. Fish Pathol. 34, 43–53.

Ragone, L.M., Burreson, E.M., 1993. Effect of salinity on infection progression and pathogenicity of *Perkinsus marinus* in the eastern oyster, *Crassostrea virginica* (Gmelin). J. Shellfish Res. 12, 1–7.

Ragone, L.M., Burreson, E.M., 1994. Characterization of overwintering infections of *Perkinsus marinus* (Apicomplexa) in Chesapeake Bay oysters. J. Shellfish Res. 13, 123–130.

Ragone-Calvo, L.M., Dungan, C.F., Roberson, B.S., Burreson, E.M., 2003. Systematic evaluation of factors controlling *Perkinsus marinus* transmission dynamics in lower Chesapeake Bay. Dis. Aquat. Org. 56 (1), 75–86. https://doi.org/10.3354/dao056075.

Ramilo, A., Carrasco, N., Reece, K.S., Valencia, J.M., Grau, A., Aceituno, P., Villalba, A., 2015. Update of information on perkinsosis in NW Mediterranean coast: identification of Perkinsus spp. (Protista) in new locations and hosts. J. Invertebr. Pathol. 125, 37–41. https://doi.org/10.1016/j.jip.2014.12.008.

Ray, S.M., 1954. Biological Studies of *Dermocystidium marinum* a Fungus Parasite of Oysters. Rice Institute pamphlet. (Monogr. Biol. Spec. Ser. Iss.), Rice Institute, Washington, DC.

Ríos, R., Aranguren, R., Gastaldelli, M., Arcangeli, G., Novoa, B., Figueras, A., 2020. Development and validation of a specific real-time PCR assay for the detection of the parasite *Perkinsus olseni*. J. Invertebr. Pathol. 169, 107301 https://doi.org/10.1016/j.jip.2019.107301.

Ríos-Castro, R., Romero, A., Aranguren, R., Pallavicini, A., Banchi, E., Novoa, B., Figueras, A., 2021. High-throughput sequencing of environmental DNA as a tool for monitoring eukaryotic communities and potential pathogens in a coastal upwelling ecosystem. Front. Vet. Sci. 8, 765606 https://doi.org/10.3389/fvets.2021.765606.

Ruano, F., Batista, F.M., Arcangeli, G., 2015. Perkinsosis in the clams *Ruditapes decussatus* and *R. philippinarum* in the Northeastern Atlantic and Mediterranean Sea: a review. J. Invertebr. Pathol. 131, 58–67. https://doi.org/10.1016/j.jip.2015.07.015.

Sagristà, E., Durfort, M., Azevedo, C., 1995. *Perkinsus* sp. (Phylum Apicomplexa) in Mediteranean clam *Ruditapes semidecussatus*: ultrastructural observations of the

cellular response of the host. Aquaculture 132, 153–160. https://doi.org/10.1016/0044-8486(94)00391-Z.

Sagristà, E., Durfort, M., Azevedo, C., 1996. Ultrastructural study of the parasite, *Perkinsus atlanticus* (Apicomplexa), on the clam *Ruditapes philippinarum*, in the Mediterranean. Sci. Mar. 60, 283–288.

Shaw, B.L., Battle, H.I., 1957. The gross microscopic anatomy of the digestive tract of *Crassostrea virginica* (Gmelin). Can. J. Zool. 35, 325–346. https://doi.org/10.1139/z57-026.

Sunagawa, S., Acinas, S.G., Bork, P., Bowler, C., Tara Oceans Coordinators, Eveillard, D., de Vargas, C., 2020. Tara oceans: towards global ocean ecosystems biology. Nat. Rev. Microbiol. 18 (8), 428–445. https://doi.org/10.1038/s41579-020-0364-5.

Villalba, A., Reece, K.S., Ordas, M.C., Casas, S.M., Figueras, A., 2004. Perkinsosis in molluscs: a review. Aquat. Living Res. 17, 411–432. https://doi.org/10.1051/alr:2004050.

Villalba, A., Casas, S.M., López, C., Carballal, M.J., 2005. Study of perkinsosis in the carpet shell clam *Tapes decussatus* in Galicia (NW Spain). II. Temporal pattern of disease dynamics and association with clam mortality. Dis. Aquat. Org. 65 (3), 257–267. https://doi.org/10.3354/dao065257.

Wijsman, J.W.M., Smaal, A.C., 2017. The use of shellfish for pre-filtration of marine intake water in a reverse electro dialysis energy plant: inventory of potential shellfish species and design of conceptual filtration systems. (Wageningen Marine Research report; No. C078/17). Wageningen Mar. Res. https://doi.org/10.18174/424555.