Development of duplex TaqMan-based real-time PCR assay for the simultaneous detection of Perkinsus olseni and P. chesapeaki in host Manila clam tissue samples

Sarah Itoïz, Morgan Perennou, Clara Mouronvalle, Evelyne Derelle, Nelly Le Goïc, Adeline Bidault, Xavier de Montaudouin, Isabelle Arzul, Philippe Soudant, Aurélie Chambouvet

Title: **Development of duplex TaqMan-based real-time PCR assay for the simultaneous detection of *Perkinsus olseni* and *P. chesapeaki* in host Manila clam tissue samples.**

Sarah Itoïz[1], Morgan Perennou[1], Clara Mouronvalle[1, 2], Evelyne Derelle[1], Nelly Le Goïc[1], Adeline Bidault[1], Xavier de Montaudouin[3], Isabelle Arzul[4], Philippe Soudant*[1] & Aurélie Chambouvet*[1]

1. Univ Brest, CNRS, IRD, Ifremer, LEMAR, F-29280, Plouzané, France
2. EPHE, PSL Research University, UPVD, CNRS, USR 3278 CRIOBE, Perpignan F-66360, France
3. Univ. Bordeaux, CNRS, EPOC, EPHE, UMR 5805, Station Marine, F-33120 Arcachon, France
4. IFREMER, Laboratory of Genetics and Pathology, Av de Mus de Loup-17390, La Tremblade, France

Running header: Itoïz *et al.*: **Duplex real-time PCR assay for the simultaneous detection of *Perkinsus* species.**

Keywords: **Alveolata, co-infection, molluscs, inhibitory effect, *Ruditapes philippinarum*, qPCR**

Corresponding author: +33 298498785; e-mails: aurelie.chambouvet@univ-brest.fr (A. Chambouvet), philippe.soudant@univ-brest.fr (P. Soudant)
* authors contribute equally to the work

Abstract

The aetiological agent *Perkinsus olseni* is globally recognised as a major threat for shellfish production considering its wide geographical distribution across Asia, Europe, Australia and South America. Another species, *Perkinsus chesapeaki*, which has never been known to be associated with significant mortality events, was recently detected along French coasts infecting clam populations sporadically in association with *P. olseni*. Identifying potential cryptic infections affecting *Ruditapes philippinarum* is essential to develop appropriate host resource management strategies. Here, we developed a molecular method based on duplex real-time quantitative PCR for the simultaneous detection of these two parasites, *P. olseni* and *P. chesapeaki,* in the different clam tissues: gills, digestive gland, foot, mantle, adductor muscle and the rest of the soft body. We firstly checked the presence of possible PCR inhibitors in host tissue samples. The qPCR reactions were inhibited depending on the nature of the host organ. The mantle and the rest of the soft body have a high inhibitory effect from threshold of host gDNA concentration of 2 $ng.\mu L^{-1}$, the adductor muscle and the foot have an intermediate inhibition of 5 $ng.\mu L^{-1}$, and the gills and digestive gland do not show any inhibition of the qPCR reaction even at the highest host gDNA concentration of 20 $ng.\mu L^{-1}$. Then, using the gills as a template, the suitability of the molecular technique was checked in comparison with the Ray's Fluid Thioglycolate Medium methodology recommended by the World Organisation for Animal Health. The duplex qPCR method brought new insights and unveiled cryptic infections as the co-occurrence of *P. olseni* and *P. chesapeaki* from *in situ* tissue samples in contrast to the RFTM diagnosis. The development of this duplex qPCR method is a fundamental work to monitor *in situ* co-infections that will lead to optimised resource management and conservation strategies to deal with emerging diseases.

1. Introduction

Manila clam, *Ruditapes philippinarum*, is extensively cultured worldwide with 4.23 million tons harvested in 2017 representing 25% of global bivalve production (FAO, 2018). Introduced in Europe in the 1970s (Flassch and Leborgne, 1994) for aquaculture purposes, this bivalve is currently the third most important bivalve produced in Europe after mussels and oysters (European Commission - DG for health and food safety, 2018). However, this valuable economic resource is regularly threatened by infectious diseases caused by a wide variety of pathogenic agents including bacteria, virus, and protists, leading to mortality events with significant economic consequences (Allam *et al.*, 2002; Jenkins *et al.*, 2013; Azevedo, 1989). It is today widely accepted that coastal ecosystems are threatened by an unprecedented environmental transformation and the influence of these changes on disease epizootics is now a major scientific issue to tackle. These drastic changes, related to multifactorial anthropogenic consequences, might alter the host-pathogen balance illustrated by an increase in prevalence and severity of disease outbreaks (Harvell, 2002; Ward and Lafferty, 2004). Perkinsosis is one of the most widespread marine diseases globally in commercially important marine molluscs including oysters, clams and abalones. Two main parasites, *Perkinsus marinus* and *Perkinsus olseni,* identified as the infectious agents of perkinsosis, have been classified as notifiable pathogens in the Organisation for Animal Health (OIE)-list diseases since 2006 (OIE, 2019a). *P. marinus* is responsible for extensive oyster mortalities mostly in the Gulf of Mexico and in the Chesapeake Bay (Mackin *et al.*, 1950; Mackin, 1951; Burreson and Andrews, 1988; Burreson *et al.*, 1994; Andrews, 1996). *P. olseni*, which has a broad geographic distribution (e.g. Europe: Arzul *et al*., 2012; Azevedo, 1989; Australia: Goggin and Lester, 1995; Asia: Hamaguchi *et al*., 1998; Park and Choi, 2001; Shamal *et al.*, 2018; America: Pagenkopp Lohan *et al*., 2018), is often associated with clam mortalities in Asia (Park and Choi, 2001; Nam *et al.*, 2018) and in Europe (Da Ros and Canzonier, 1985; Ruano and Cachola, 1986; Azevedo, 1989; Pretto *et al.*, 2014), and abalone mortality in Australia (Goggin and Lester, 1995). Even when infection by *Perkinsus* species does not directly produce host death it can induce sublethal effects resulting in reduced growth and reproductive activity and a general decrease in host health which favours secondary opportunistic infections (Tall *et al.*, 1999; Dittman *et al.*, 2001; Montes *et al.*, 2001; Lee *et al.*, 2001).

The advent of molecular methods brought new insights in species definition and helped unravel cryptic infections across various fields including marine environments (Putaporntip *et al.*, 2009; Chambouvet *et al.*, 2015). Recently, using a combination of culture methodology and conventional PCR, co-occurrences of two *Perkinsus* species within the same host tissue samples have been reported (e.g. Reece *et al*., 2008, Coss *et al.*, 2001, Takahashi *et al*., 2009). In Europe, two *Perkinsus* species, *P. olseni* a*nd P. chesapeaki,* were identified sporadically co-infecting the native grooved carpet shell clam, *Ruditapes decussatus,* from Leucate lagoon (Arzul *et al.*, 2012) and the exotic Manila clam, *R. philippinarum,* from Galicia (NW Spain)(Ramilo *et al.*, 2016). Hypothetically, *P. olseni* and *P. chesapeaki* were accidentally introduced with their vector, the Manila clam *R. philippinarum* from Asia and the soft-shell clam, *Mya arenaria*, or the hard clam, *Mercenaria mercenaria,* from U.S.A., respectively (Arzul *et al.*, 2012). As opposed to *P. olseni, P. chesapeaki* has never been obviously associated with a mass mortality event despite some suspicions it may cause mortality in the stout razor clam, *Tagelus plebeius,* in the U.S.A. (Bushek *et al.*, 2008). Although these two parasites are detected along the European Atlantic coastline, their distribution, prevalence and impact on infection outcome remain an important scientific gap that urgently needs to be fulfilled because the 'one parasite-one disease' paradigm is now outdated. Indeed, today, disease outcomes frequently reflect an interaction network including numerous different pathogens within a single host (Bass *et al.*, 2019).

The standard methodology for *Perkinsus* spp. quantification, recommended by the O.I.E., is the incubation of infected host tissues samples in Ray's fluid thioglycolate medium (RFTM) followed by alkaline digestion with NaOH and blue-black staining of the infectious parasitic cells with Lugol's iodine (Choi *et al*., 1989; OIE, 2019b; Ray, 1952). The RFTM methodology, which is easy to perform, inexpensive and sensitive to a very low infection intensity, is now widely used (Choi *et al.*, 1989; Bushek *et al.*, 1994). In parallel, classical haematoxylin and eosin staining histology methodology, also recommended by the OIE, is very useful to visualise lesions and parasite distribution in tissues even if it is time-consuming and less sensitive (OIE, 2019b). It allows a qualitative detection of parasites from *Perkinsus* genus within the different tissue samples. However, these two methods do not adequately identify the parasite species or the presence of co-infection by parasites of the same genus. These two non-specific methods should now be complemented with molecular tools for quantitative and

qualitative detection (Almeida *et al.*, 1999; Novoa *et al.*, 2002; Audemard *et al.*, 2008; Balseiro *et al.*, 2010). Molecular based methods, e.g. conventional PCR and RFLP (Restriction Fragment Length Polymorphic) assays, were used to detect the presence or absence of the genetic signature of the different *Perkinsus* species in culture or in host tissue samples (Takahashi *et al.*, 2009; Arzul *et al.*, 2012; Ramilo *et al.*, 2016). Recently, real-time quantitative PCR (qPCR) methodology was developed to quantify the abundance and the prevalence of different *Perkinsus* species within host tissue samples and / or in environmental samples (Audemard *et al.*, 2004; Umeda and Yoshinaga, 2012; Ríos *et al.*, 2020). In 2018, Cui *et al.* highlighted for the first time, using qPCR method, a seasonal pattern between *P. olseni* and *P. beihaiensis* infecting the clam *Soletellina acuta* in China (Cui *et al.*, 2018). Such results highlighted that cryptic infection by different *Perkinsus* species, usually unnoticed by classical methodology, can play a key role in the infection process and therefore on disease outcomes.

The qPCR methodology is an accurate and sensitive tool which should be more widely used because it is now clear that co-infection between *Perkinsus* species might be more frequent than expected. However, a major drawback for qPCR assays is the presence of potential PCR inhibitors that can hinder parasite quantification. These inhibitor compounds, usually co-extracted during the nucleic acid extraction, are currently prevalent in shellfish tissue (Hohweyer *et al.*, 2013). To avoid strong PCR inhibition, the dilution of inhibited samples provides a rapid and simple way to overcome this problem (Renault *et al.*, 2000). This easy method was performed to rule out false negatives but may dilute the number of targeted molecules below the limit of the detection method (Batista *et al.*, 2007).

Given that *P. olseni* is not systematically associated with mortality, the detection and quantification of another infectious agent co-infecting same hosts could lead to a better comprehension of the disease outcome. The aim of this study was to develop a duplex TaqMan-based real-time PCR assay to quantify the two *Perkinsus* species, *P. olseni* and *P. chesapeaki,* in Manila clam host tissues samples and thus detect and monitor specifically the co-infection of *P. olseni and P. chesapeaki* in the valuable population of Manila clams from different locations.

2. Material & methods

2.1. *Manila clams* sampling

Clams were collected during a sampling campaign in Arcachon bay (SW France, Atlantic coast, 44°41'60" N;1°10' W) the 7th and 8th of November 2018 (Itoïz *et al*., in prep). A total of 55 Manila clams, *Ruditapes philippinarum*, were collected from the station Lanton (Figure S1; Table S1). Arcachon bay is a mesotidal lagoon of 180 km^2 where intertidal mudflat macrofauna is mainly dominated by *R. philippinarum*, which could represent up to 90% of the total biomass (Bertignac *et al.*, 2001).

Of the 55 clams sampled, fifty were dissected for RFTM and molecular assays. Briefly, clams were opened on ice and one gill was weighed and incubated in RFTM supplemented with antibiotics (penicillin 66 $\mu g.mL^{-1}$ and streptomycin 32 $\mu g.mL^{-1}$) and antimycotic (nystatin 0.04 $mg.mL^{-1}$), with subsequent *Perkinsus* quantification according to Choi *et al.* (1989). The rest of each oyster was dissected into six compartments: the second gill, the digestive gland, the mantle, the adductor muscle, the foot and the remaining tissue. Each organ was weighed and preserved separately in 80% ethanol at 4°C until further molecular analysis. Shells were measured and weighed. Five clams were kept in a 40L tank of filtered (0.2 µm) sea water at 15°C for 10 days before using them for establishing *P. olseni* and *P. chesapeaki* cultures.

2.2. Ray's fluid thioglycolate medium (RFTM)

Body-burden assays were conducted following the RFTM method as recommended by the O.I.E. (Ray, 1952; Choi *et al.*, 1989; OIE, 2019b). Briefly, after incubation in RFTM in the dark for five days at room temperature, allowing the enlargement of trophozoites (hypnospore formation) (Ray, 1952), gill tissue samples were digested with 2M NaOH solution for 3 h at 60 °C preserving the hypnospore cell structure (Choi *et al*., 1989). Hypnospores were then stained with a Lugol's iodine solution (4 %), and counted in a

Nageotte chamber (ten lines in triplicate) under a microscope (Leica DM-IRB; x10 magnification). The counting results were expressed as the number of hypnospores per gram of wet tissue.

2.3. *In vitro* culture of monoclonal strains of *P. olseni* and *P. chesapeaki*

The five preserved Manila clams were used to established cultures following a simplified protocol from Casas *et al.* (2002a). In short, gill tissue samples were excised and decontaminated with five successive baths of antibiotic solution (concentrations per L^{-1}: 400 000 U ampicillin - 0.4 g streptomycin sulphate (Sigma-Aldrich), 0.4 g kanamycin (Sigma-Aldrich), 0.2 g gentamycin (Sigma-Aldrich)) and baths of sterile sea water. Gill samples were incubated in *Perkinsus* medium broth (ATCC medium 1886) supplemented with antimycotic (40 $U.mL^{-1}$ nystatin, Sigma-Aldrich) and antibiotics (100 $U.mL^{-1}$ penicillin and 0.1 $mg.mL^{-1}$ streptomycin, Sigma-Aldrich) at 25°C for 10-15 days to allow trophozoites to proliferate. Five cultures were initiated from the five gills that were initially incubated. To characterise the *Perkinsus* species, the five cultures were sampled after two weeks of incubation for molecular analysis based on PCR (using primers ITS-85/ITS-750, Casas *et al.*, 2002b) and amplicon sequencing. Monoclonal cultures of *P. olseni* and *P. chesapeaki* were established using two of these cultures. Briefly, using the agar-based method developed by Cold *et al*. (2016), initial cultures of *P. olseni* and *P. chesapeaki* were diluted at 2,000 $cells.mL^{-1}$ in *Perkinsus* medium broth (ATCC medium 1886) before plating onto solid medium (*Perkinsus* medium broth [ATCC medium 1886] implemented with 0.75 % agar). Agar plates were incubated at 25°C until the first visible colonies appeared. Each colony was assessed using an inverted light microscope and sub-cloned twice using the same methodology before seeding into liquid *Perkinsus* broth medium (ATCC medium 1886). Two monoclonal strain cultures were selected to establish *P. olseni* and *P. chesapeaki* plasmid-standard curves (Figure S2).

2.4. DNA extraction

The genomic DNA (gDNA) of the six organs (gill, digestive gland, mantle, adductor muscle, foot and the remaining tissue samples) was extracted using CTAB-based DNA extraction method adapted from Winnepenninckx *et al.* (1993). Organ tissues were

transferred in bead beating tubes containing three different bead sizes (2.8 mm, 1.4 mm and 0.1 mm of diameter, Ozyme) with 1 mL of CTAB extraction buffer (2 % CTAB, 100 mM TrisHCl pH=8.0, 20 mM EDTA, 1.4 mM NaCl) and placed into cooling rack. Samples were then ground and homogenized following two bead beating cycles (45 seconds of bead beating at 6 m. s^{-1} following by 20 seconds stopped) using the FastPrep-24 5G (MP Biomedicals). After this step, β-mercaptoethanol (0.2 %) and proteinase K (0.1 $mg.mL^{-1}$) were then added in each tube and samples were incubated for 30 min at 60°C. The one exception to this was foot tissue samples, which were incubated for 12 hours due to this tissue's dense muscular structure. Lysates were mixed in chloroform/isoamylalcohol (24:1, v/v) and emulsified before spinning for 10 min at 18.000 g rpm in a cooled microcentrifuge (4°C). This step was repeated twice for the foot tissue samples. Aqueous phases were treated with RNAse solution (10$mg.mL^{-1}$, Sigma-Aldrich) for 30 minutes at 37°C, and then DNA was precipitated with cold 100% isopropanol overnight at 4°C. DNA was pelleted and rinsed with two successive washes of cold 70% ethanol. The DNA pellets were then dried at room temperature until complete ethanol evaporation and resuspended in 300 µl of pure molecular grade water (Corning). After DNA quantification using the Qubit dsDNA HS assay kit (Invitrogen), the DNA samples were stored at -20 °C until further downstream processing.

2.5. Duplex qPCR assay development

2.5.1. Targeted amplification of the ITS sequences of two *Perkinsus* monoclonal strains

PCR assays were performed using *Perkinsus*-genus specific primers ITS-85/ITS-750 (Casas *et al.*, 2002b) targeting the sequences of rDNA Internal Transcribed Spacer (ITS) regions. All PCR reactions were performed (25 µL final volume) using GoTaq Polymerase G2 (Promega) as described by Promega including: 1X green GoTaq reaction buffer (Promega), 0.2 mM of each dNTP, 0.5 µM of each primer and 1.25 U of GoTaq DNA polymerase (Promega). After a first denaturation step of 5 min at 95°C, thirty-five cycles of three steps were carried out as follows: denaturation for 30 s at 95°C, annealing for 30 s at 56°C and extension for 45 s at 72°C. After a final extension for 10 min at 72 °C and a last

step at 4 °C, amplified PCR products were checked on a 1% agarose gel and purified directly using Wizard SV Gel and PCR clean-up System (Promega) as recommended by the manufacturer. The two purified amplicons were double-strand sequenced using ITS-85/ITS-750 primers. Species identity of the two monoclonal cultures was confirmed by submitting both sequences to the NCBI non-redundant (nr) database (BLASTn) (Figure S2). Both sequences were deposited in GenBank (accession no. MW187111 for the *P. olseni* monoclonal culture and MW187112 for the *P. chesapeaki* monoclonal culture).

2.5.2. Production of specific *P. olseni* and *P. chesapeaki* plasmid standards

Plasmid standards are a useful resource for relative quantification of parasite in host tissue. The methodology used to establish the two standards was based on the duplex study between *Perkinsus* and *Haplosporidium* species from Xie *et al.* (2013). ITS sequences were cloned using the pGEM-T easy vector system I (Promega) and transformed into homemade competent *Escherichia coli* XGold following the manufacturer's recommendations. Clones were blue/white screened and numerous clones per library were selected for overnight growth in Lysogeny Broth media (LB) at 37°C. Plasmid DNA was purified using the NucleoSpin Plasmid kit (Macherey-Nagel) and quantified using the Qubit™ dsDNA HS assay kit (Invitrogen). Plasmid count was obtained using the following formula: $molecule/\mu l = a / (3\ 665\ bp \times 660\ Da) \times 6.022 \times 10^{23}$ where a is the DNA plasmid concentration, 3665 bp is the full-length plasmid including the vector (3015 bp) and the amplified fragment (650 bp), 660 Da is the molecular weight of one base pair and 6.022×10^{23} is the Avogadro molecular constant.

2.5.3. Design of species-specific primers and TaqMan hydrolysis probes

With their high genetic polymorphism allowing species-level discrimination, ITS regions have been commonly selected as good candidate regions for species-specific primer development (Casas *et al.*, 2002a; b; OIE, 2019b). Primers and probes were designed using Primer3Plus (qPCR settings) (Untergasser *et al.*, 2012) with GenBank-accessed template sequences JQ669642, KX514117, KX514123, LC431768, DQ516714, MG733367, MG733365, KP764683, KX514103, KP764681, FJ481986, and EU293848 for

P. olseni, and EU919479, EU919489, EU919501, AY876312, AY876314, EU919465, EU919496, AY876318, AY876316, MF186901, MF186913, and MF186910 for *P. chesapeaki*. To optimise the duplex real-time PCR, sets of primers and probes were designed with similar annealing temperatures (Tm) and amplicon lengths (Figure 1; Table 1). Specificities were assessed *in silico* using (1) ARB Software (Ludwig *et al.*, 2004) against a multiple sequence alignment of *Perkinsus* ITS1- 5.8S – ITS2 region constructed from sequences available on GenBank (accessed March 2019) (Table S2); (2) Primer-BLAST (accessed March 2019) against the NCBI nonredundant (nr) DNA database (available at https://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Table S3); and (3) Blastn submitting probes sequences to the NCBI nr database (available at blast.ncbi.nlm.nih.go) (Table S4). Secondary structure and dimer formation were checked using the OligoEvaluator (Sigma-Aldrich).

2.5.4. Real-time PCR assays development

Real-time PCR assays were performed with the LightCycler 480 Probes Master (Roche) specifically developed for the hydrolysis probes reaction using the LightCycler 480 II (Roche).

The specificity of primers and probes were firstly tested using gDNA of the two monoclonal strains of *P. olseni* and *P. chesapeaki*. Gill gDNA from an uninfected clam was used to confirm the absence of hybridisation with the host matrix. For all conditions, a negative control (pure water) was added to verify the non-contamination of the qPCR mixture and the formation of chimeras. All gDNA concentrations were adjusted to 1 pg using the Qubit dsDNA HS assay kit (Invitrogen).

All qPCR assays were performed as recommended by the supplier LightCycler 480 Probes Master (Roche). Primers and probes were respectively diluted in pure water at 50 µM and 2 µM to reach the recommended working solution concentration. The mix LightCycler 480 Probes Master 2X conc. (Roche) contains FastStart Taq DNA Polymerase, reaction buffer, dNTP mix (with dUTP instead of dTTP) and 6.4 mM $MgCl_2$. The reaction volume for duplex qPCR consisted of 1X LightCycler 480 Probes Master premixed, 0.2 µM of primer PolsITS2-F, 0.2 µM of PolsITS2-R, 0.2 µM of PchesITS2-F, 0.2 µM of PchesITS2-R, 0.5 µM of PolsITS2-probe (FAM), 0.5 µM of PchesITS2-probe

(LC640), and 5 µL of DNA sample with adjusted concentration. The reaction volume for simplex qPCR consisted of 1X LightCycler 480 Probes Master premixed, 0.2 µM of primer PolsITS2-F or PchesITS2-F, 0.2 µM of PolsITS2-R or PchesITS2-R, 0.5 µM of PolsITS2-probe or PchesITS2-probe, and 5 µL of DNA sample with adjusted concentration. The thermal cycling conditions were as follows: 5 min of pre-incubation at 95 °C followed by 45 cycles of amplification containing 10 s of denaturation at 95°C and 20 s of annealing at 55°C. At the 72°C extension step, for each cycle a single fluorescence acquisition was recorded for the following filter combination: FAM (465-510), VIC/HEW/Yellow555 (533-580) and Cy5/Cy5.5 (618-660). The PCR assay was finally terminated by cooling for 2 min at 40°C. In each qPCR run, samples were run in triplicate and two negative controls containing non-infected Manila clam gDNA and pure molecular grade water were included. Triplicates were averaged for each sample. If one or two values of a triplicate were anomalous, the sample was reprocessed to discard a potential manipulation error.

2.5.5. Repeatability of the duplex qPCR method

The variation of the duplex qPCR method was evaluated depending on the repeatability of the 10-fold dilution plasmid standards of *P. olseni* and *P. chesapeaki* from different qPCR runs. The coefficient of variation (CV) was calculated as follows: $CV = \frac{sd}{\bar{x}} \times 100\%$ (sd: standard-deviation and $\bar{x}$: mean related to one plasmid dilution) for each dilution ($2.5x10^1$ to $2.5x10^6$ total copies) of the reference standards. For each dilution, the means of Ct values and standard deviations were calculated from six different qPCR runs. The coefficients of variation were then calculated for all plasmid concentrations.

2.6. Inhibitory effect of host tissue on the qPCR assay

First, standard curves (supplemented with water) were crossed and determined by duplex qPCR assay to establish reference values of non-inhibited reactions for both species. The sensitivity of the duplex qPCR was estimated on plasmid-inserted ITS1-5.8S-ITS2 of *P. olseni* and *P. chesapeaki* with 10-fold serial dilutions from $2.5x10^1$ to $2.5x10^6$ total copies in triplicates. Sensitivity is represented by two parameters: 1) the

efficiency (E), which is the evaluation of the fraction of target molecules that are copied in one PCR cycle, where 100% represents an optimal doubling amount of DNA; and 2) the limit of detection (LOD), which is the lowest plasmid concentration giving positive and homogeneous Ct (Cycle threshold) values. Standard curves were represented by plotting the logarithm of the plasmid copy number against the corresponding measured Ct value. Thus, the qPCR efficiency (%) was calculated, depending on the slope of each curve of serial diluted target, as follows: $E = (10^{(-1/slope)} - 1) \times 100\%$. The detection limit (number of total copies) was determined as the lowest concentration of plasmids within the linear range showing significant and homogeneous amplification signal in triplicate using the LightCycler 480 v. 1.1.1.62 software (Roche).

Then, qPCR assays were carried out to determine the effects of the host gDNA organ type (i.e. gill, digestive gland, adductor muscle, foot, mantle and the remaining tissue) on the sensitivity of the qPCR duplex reaction. Based on RFTM and qPCR assay results, three *Perkinsus*-free clams were selected to determine the possible inhibitory effect for each organ type. To assess a global trend of organ influence, the mean Ct value and the standard errors were calculated from the three *Perkinsus*-free individuals for each concentration. Parameters of sensitivity for the two assays were determined in duplex reaction on crossed 10-fold serial dilutions of *P. olseni* and *P. chesapeaki* plasmids DNA ($2.5x10^1$ to $2.5x10^6$ total copies) supplemented with a dilution (2, 5, 10, and 20 $ng.\mu L^{-1}$) of host gDNA organ. In each qPCR assay, inhibitor-free standard curves (without host gDNA extract) were added and Ct values were measured.

Standard dilution curves supplemented with extracted of organ gDNA were represented by plotting the logarithm of the plasmids copy number against the corresponding measured Ct value. Standard curves (plasmids only) determined above were plotted as an organ-free reference for each duplex qPCR to compare possible inhibition effect of each organ on the value of efficiency (%). The qPCR efficiency of the reaction for each condition was calculated from each standard curve as described above. The detection limit (number of total copies) was determined as the lowest concentration of plasmids within the linear range showing significant and homogeneous amplification signal in triplicate using LightCycler 480 v. 1.1.1.62 software.

2.7. Comparison of the duplex qPCR and the RFTM method on gill tissue

Comparison of RFTM and qPCR methodologies on gill tissue (the most currently targeted tissue for perkinsosis detection) is essential to validate the molecular assay developed in this study. To compare perkinsosis results obtained with the standard RFTM method and the duplex real-time PCR method, all 50 gill DNA samples were tested by duplex qPCR. To ensure a reliable comparison between infected individuals, the concentrations of all gill DNA samples were adjusted to 20 $ng.\mu L^{-1}$ as determined above. The qPCR was then run in triplicate using the same parameters as above. The 10-fold dilution plasmid standards for *P. olseni* and *P. chesapeaki* were added to the run to determine copy concentrations in gill tissue. The mean copy number per sample was automatically calculated by the LightCycler 480 v. 1.1.1.62 software. The intensity of infection was calculated from the number of copies detected divided by the wet weight of the corresponding gill for each species of parasite.

2.8. Statistical analysis

The inhibitory effect of organ gDNA extracts at several concentrations (20, 10, 5, 2 $ng.\mu L^{-1}$) were compared to determine which concentrations represented the best dilution to optimise *Perkinsus* detection in tissue. Each linear curve was compared to the standard 10-fold plasmids dilution by a one-way analysis of covariance (ANCOVA). The majority of variables tested for each inhibitory condition fulfilled assumptions for application of ANCOVA. Plasmid concentrations were log-transformed. A regression model was fitted (Rainbow test, p-value > 0.05). Residuals were independent (Durbin-Watson test, p-value > 0.05) and normally distributed (Shapiro-Wilk test, p-value > 0.05). Homogeneity of variances was verified (Bartlett test, p-value > 0.05). The ANCOVA was used to evaluate the effect of organ on Ct values depending on organ concentration (covariate). Curves showing no significant differences from the standard 10-fold plasmids dilution were selected as good candidates for *Perkinsus* detection. Absence of difference from the standard demonstrated a reaction non-inhibited by host DNA extract. If several curves showed no significant difference, the highest concentration of organ DNA was adopted to optimise *Perkinsus* detection.

The agreement between the duplex qPCR assay and the RFTM assay to measure infection intensity was evaluated by descriptive parameters and statistical analysis. *Perkinsus* prevalences (proportion of infected individuals to the total number of hosts sampled, %) were determined for both methods and the species was determined when possible. Two parameters, the concordance and the discordance, were adapted for this study from Langton *et al.* (2002) and calculated. The concordance, calculated by counting pairs of positive samples and pairs of negative samples, is the percentage of chance that an identical sample analysed by two different methodologies will have the same result. The discordance, calculated by counting the unpaired samples (i.e. positive by RFTM and negative by qPCR assay, and vice versa), is the percentage chance that an identical sample analysed by two different methodologies will have the different result. The relationship between both methods was tested using a linear model and the Spearman correlation coefficient on infection intensities from qPCR and RFTM positive individuals.

Finally, the mean infection intensities determined by both methods were compared to verify their congruence. The infection intensities related to plasmid quantifications were evaluated in number of cells per gram of wet gill with the relationship established between the qPCR assay and the RFTM assay ($y=0.60x+4.36$; y: log of qPCR-infection intensity in number of copies.g of wet gill^{-1}; x: log of RFTM-infection intensity in number of cells.g of wet gill^{-1}).

3. Results

3.1. Specificity of the PCR assay

Primer and probe design is a crucial step for an accurately duplexing real-time PCR assay. *In silico* tests demonstrated that primers and probes were dissimilar in sequence to closely related *Perkinsus* species sequences and other important parasitic or heterotrophic protist families described in clam populations and in surrounding environments (Table S2, S3, S4).

Molecular tests were conducted by comparing the same amount (1 pg) of *P. olseni* and *P. chesapeaki* gDNA extracted from monoclonal cultures. For the *P. olseni* primers (PolsITS2_F/PolsITS2_R) and probe (PolsITS2_probe) set, amplification (Ct: 26.4 ± 0.07) was detected using *P. olseni* gDNA as a template with the filter FAM (465-510 wavelengths) corresponding dye excitation wavelength coupled with *P. olseni* probe (Figure 2). As expected, no amplification was detected for all wavelengths with *P. chesapeaki* gDNA, host gDNA or pure water as template.

The *P. chesapeaki* primers (PchesITS2_F/PchesITS2_R) and probe (PchesITS2_probe) set amplified (Ct: 26.7 ± 0.09) only *P. chesapeaki* gDNA with the suitable filter LC640 (618-660 wavelengths), and no amplification was detected for other wavelengths or other templates, *P. olseni* gDNA, host gDNA and pure water (Figure 2). These results demonstrate that the qPCR primers and probes sets are specific to the targeted gDNA without any cross amplification.

3.2. Standards PCR sensitivity and repeatability

Given the unknown number of ribosomal operons in parasite genomes and the ploidy and nuclear count of each life stage, plasmids are useful as a reproducible and stable standard for qPCR assays (Dhanasekaran *et al.*, 2010). The ITS sequence of each parasite culture was isolated via plasmid purification from cloned and transformed competent bacteria. Two standard curves (*P. olseni*: $y = -3.33x + 39.49$; *P. chesapeaki*: $y = -3.38x + 40.29$; Figure 3) were generated from 10-fold serial dilutions from 2.5×10^1 to 2.5×10^6 copies of ITS rDNA plasmid. Ct values of both standard curves showed strong linear correlations with the targeted template (*P. olseni*: $R^2 = 0.95$; *P. chesapeaki*: $R^2 = 0.95$; Figure 3). The limit of detection was determined to be 25 copies of the target DNA in a 10-µL reaction volume; below this number of plasmid copies (2.5 copies; data not shown), triplicates displayed non-replicable Ct values. DNA amplification was detected within the range of 2.5×10^1 to 2.5×10^6 plasmids with Ct values ranging from 34.9 ± 1.6 to 18.8 ± 1.5 and to 35.1 ± 1.6 to 18.4 ± 1.5 for *P. olseni* and *P. chesapeaki* respectively. The efficiency of each set of primers is evaluated from standards at 99.8% for *P. olseni* and 97.8% for *P. chesapeaki* (Figure 3). The theoretical maximum of 1.0 (or 100%) indicates that the amount of product doubles with each cycle (Bustin *et al.*, 2009).

The coefficient of variation (CV) for the duplex qPCR method was estimated for *P. olseni* standard between 3 to 8% (Table 2).

3.3. Inhibitory effects of host tissue gDNA extract on the duplex qPCR assays

Organ gDNA extract may have inhibitory effects on the efficiency of the qPCR amplification. To test this organ-dependent qPCR effect, the sensitivity and the inhibition of the duplex qPCR assay were evaluated according to the type of organ (gill, digestive gland, adductor muscle, foot, mantle and remaining tissue) at different concentrations of gDNA (2, 5, 10, 20 $\mu g.\mu L^{-1}$). Ct values varied from *P. olseni* and *P. chesapeaki* standards depending on organ type and gDNA concentration used for the amplification (Figure 4). Results of ANCOVA and efficiencies are synthesised in Table S5. Efficiencies (E) between 90% and 110% are tolerated for a complex host matrix even if slight over- and under-estimation can happen.

3.3.1. Effect of organ gDNA extracts on *P. olseni*-plasmid detection

P. olseni-standards containing only plasmids represented the positive control with an amplification of *P. olseni* gDNA in absence of inhibitory effects (Figure 3). For gill and digestive gland, the four concentrations did not influence the linear 10-fold dilution curves (ANCOVA, p-value >0.05) (Figure 4A). With no difference observed from the standard curve, the maximum concentration is 20 $ng.\mu L^{-1}$ for gDNA of both gills (efficiency (E) = 100.3%) and digestive gland (E = 100.4%). For the adductor muscle, only the amplification curve corresponding to 20 $ng.\mu L^{-1}$ showed a significant difference from the *P. olseni* standard (ANCOVA, F=5.53, p-value < 0.05). It had a high efficiency of 115.4 %. Maximum concentrations showing no difference from the standard were the 10 $ng.\mu L^{-1}$ and 5 $ng.\mu L^{-1}$ curves (ANCOVA, p-value >0.05). The 5 $ng.\mu L^{-1}$ curve was preferred for its optimal efficiency of 98.7%. For foot, the amplification curves corresponding to 10 $ng.\mu L^{-1}$ and 20 $ng.\mu L^{-1}$ showed significant differences from the *P. olseni* standard (ANCOVA, $F_{10ng/\mu L}$=8.4, $p\text{-value}_{10ng/\mu L} < 0.01$; $F_{20ng/\mu L}$=17.6, $p\text{-value}_{20ng/\mu L} < 0.001$). The maximum concentration with no difference from the standard was the 5 $ng.\mu L^{-1}$ curve (E = 92.8% ; ANCOVA, p-value >0.05). For mantle, the 2 $ng.\mu L^{-1}$ was the maximum concentration that did not alter the linear 10-fold dilution curve (E = 92% ; ANCOVA, p-

484 value >0.05). Even if the amplification curves for 5 ng.µL^{-1} and 20 ng.µL^{-1} showed non-
485 significant differences from the standard, their shifts are out of the error range, which
486 highlights an important variability for these concentrations (Figure 4A). For the
487 remaining tissue, 2 ng.µL^{-1} was the maximum concentration of gDNA that did not alter
488 the linear 10-fold dilution curve (E = 105.6% ; ANCOVA, p-value >0.05). Other
489 concentrations showed significant differences or efficiencies too high (e.g. the 10 ng.µL^{-1}
490 curve: E = 121.7%) to be selected.

491

492 **3.3.2. Effect of organ gDNA extracts on *P. chesapeaki*-plasmids detection**

493 Same trends are observed for *P. chesapeaki* where standard containing only plasmids
494 represented the positive control without any inhibitory effects (Figure 3). For gill and
495 digestive gland, the addition of gDNA extract did not influence the linear 10-fold dilution
496 curves at any of the tested concentrations (ANCOVA, p-value >0.05) (Figure 4B). With no
497 difference observed from the standard curve, the maximum concentration was 20 ng.µL^{-1}
498 for gDNA of both gills (E = 98.3%) and digestive gland (E = 97.2%). For the adductor
499 muscle, the curve corresponding to 10 ng.µL^{-1} showed a significant difference from the
500 standard (ANCOVA, $F_{10ng/\mu L}$=5.5, p-value < 0.05). The maximum concentration showing
501 no difference from the standard was the 5 ng.µL^{-1} curve (E = 96.3% ; ANCOVA, p-value
502 >0.05). For foot, the gDNA concentrations of 10 ng.µL^{-1} and 20 ng.µL^{-1} showed significant
503 differences from the *P. chesapeaki* standard (ANCOVA, $F_{10ng/\mu L}$= 26.6, p-value$_{10ng/\mu L}$
504 <0.001; $F_{20ng/\mu L}$= 16.1, p-value$_{20ng/\mu L}$ <0.001). The maximum concentration of foot gDNA
505 extract with no difference from the standard was the 5 ng.µL^{-1} curve (E = 89.3%;
506 ANCOVA, p-value >0.05) even if the efficiency indicated a slight underestimation of the
507 target. For mantle, 2 ng.µL^{-1} was the maximum concentration that did not alter the linear
508 10-fold dilution curve (E = 92.1%, ANCOVA, p-value >0.05). The curve corresponding to
509 5 ng.µL^{-1} showed a non-significant difference from the standard but was discarded due
510 to a lower concentration threshold for *P. olseni* (2 ng.µL^{-1}). The host tissue gDNA
511 concentration of 2 ng.µL^{-1} was the safest trade-off showing no difference from the
512 standard. For the remaining tissue, the 2 ng.µL^{-1} was the maximum concentration of
513 gDNA that did not alter the linear 10-fold dilution curve (E = 95.7%; ANCOVA, p-value
514 >0.05).

3.4. Comparison of two quantitative methods: RFTM and qPCR duplex on gill tissue samples

To evaluate the effectiveness of the qPCR assay for detecting and quantifying P. *olseni* and *P. chesapeaki* levels in clam gill, the copy number estimated with this method for each prevalent species was determined and compared with the RFTM infection intensities.

Based on the previous results described above, only Ct values within the standard range of $2.5x10^1$ to $2.5x10^6$ total copies were considered positive. Therefore, all values below this range were considered as unquantifiable. No samples were quantified above $2.5x10^6$ total copies. The total prevalence of *Perkinsus* estimated by RFTM assay and qPCR assay was 74%, representing a total of 37 infected clams over the 50 clams tested (Table 3). Prevalence of *P. olseni* single infections represented 68% (34 clams) of sampled individuals whereas co-infection represented only 6% (3 clams). Among the co-infected hosts (n=3), the *Perkinsus* spp. mean infection intensity was $1.37x10^4 \pm 2.14x10^4$ cells. g^{-1} of wet tissue. Based on paired and unpaired prevalences from both RFTM and qPCR assays, the concordance was estimated at 88% and the discordance at 12% (Table 4). The three discordant individuals, positive by RFTM assay and negative with qPCR assay, showed very light infection intensities ranging from 8.1 cells.g^{-1} of wet tissue to $6.0x10^2$ cells.g^{-1} of wet tissue.

To determine the relationship between both quantitative methods, only positive clams by RFTM and qPCR were compared (Figure 5). A linear regression was determined for the qPCR-infection intensity and the RFTM-infection intensity relationship ($y=0.60x+4.36$, adjusted-$R^2=0.61$, n=34). A significant Pearson's coefficient was obtained for the qPCR-infection intensities and the RFTM-infection intensities ($r=0.79$, p-value<0.001) showing a positive correlation. The infection intensity (in cells.g^{-1} of wet tissue) for each individual was evaluated from the qPCR assay following the relationship described before ($y=0.60x+4.36$; Figure 5). A discrepancy from the linear regression line was observed for low infection values (below 100 cells.g^{-1} of wet gill). To optimise parasite detection, negative individuals and mismatched individuals were discarded. Consequently, the intercept of this linear relationship is not equal to zero which

548 introduces an overestimation of infection intensity. The mean infection intensity
549 estimated by qPCR assay was $3.35x10^5 \pm 8.55x10^5$ cell. g^{-1} of wet tissue. Separately,
550 mean infection intensity for *P. olseni* was estimated at $1.37x10^4 \pm 2.14x10^4$ cells.g^{-1} of
551 wet tissue and for *P. chesapeaki* at $2.50x10^1 \pm 2.6910^1$ cells.g^{-1} of wet tissue. The mean
552 proportion of *P. olseni* was 9% ± 5%.
553
554
555 **4. Discussion**
556
557 **4.1. The RFTM assay lacks specificity**
558 Almeida *et al*. (1999) suggested that planktonic organisms like dinoflagellates could
559 react positively in RFTM assays, highlighting a lack of genus-specificity when used on
560 environmental samples by inadequately expert diagnosticians. This standard
561 methodology also lacks species specificity, hypnospores showing no distinctive
562 morphological traits, and furthermore does not detect all *Perkinsus* parasites: *P. qugwadi*
563 does not form hypnospores in RFTM and thus may not be detected by the assay (Itoh *et*
564 *al.*, 2013). Using only this methodology allows estimation of the prevalence and
565 intensities of "generic" *Perkinsus* infection, acceptable when only a single species is
566 present, but it will miss cryptic co-infections, as noted for *P. marinus* and *P. chesapeaki* in
567 Chesapeake Bay (Reece *et al.*, 2008).
568 Using the RFTM methodology, we observed 74% prevalence with a strong infection
569 intensity of $9.04x10^4 \pm 2.24x10^5$ cells.g^{-1} of wet tissue, is consistent with the results
570 obtained by Dang *et al.* (2010) from Arcachon Bay in November 2006 and 2007 where
571 prevalences were 75% and 70% and infection intensities of $1.58x10^5$ and $7.94x10^4$
572 cells.g^{-1} of wet tissue respectively. However, the diversity of *Perkinsus* species occurring
573 in Arcachon Bay is yet not resolved. From a previous sampling campaign carried out in
574 October 2017 in Arcachon Bay (data not shown), *Perkinsus* culture cell lines were
575 established from clam gill samples. The genetic diversity in the ITS1-5S-ITS2 rDNA
576 region revealed that 17 cultures (of n = 22) were 100% similar in sequence to *P. olseni*
577 across 627 bp, while 5 cultures were 97-100% similar to *P. chesapeaki* across 639 bp. All
578 these results are congruent with previous studies showing co-occurrence of these two
579 *Perkinsus* species in European coastal environments (Arzul *et al.*, 2012; Ramilo *et al.*,
580 2016). Hence, the *in situ* occurrence of these cryptic infections in the Arcachon Bay has

made necessary the development of a non-culture based method like the duplex qPCR to distinguish between two parasites, *P. olseni* and *P. chesapeaki*, in Manila clam tissue samples.

4.2. The real-time PCR improved our conception of *in situ* infection

Real-time PCR methodology has largely proven to be successful for the diagnose of *in situ* infection by protists, viruses or bacteria (Hardegen *et al.*, 2010; Kuhar *et al.*, 2013; Duffy *et al.*, 2013). In context of emerging diseases in aquaculture, it brings a new perspective on species occurrence and intensity of infection (Gauthier *et al.*, 2006; Ulrich *et al.*, 2007; Umeda and Yoshinaga, 2012; Cui *et al.*, 2018). Detection of perkinsosis has been accomplished using two types of real-time PCR chemistry, SYBR Green (Audemard *et al.*, 2004; Ulrich *et al.*, 2007) and TaqMan (Gauthier *et al.*, 2006; Marquis *et al.*, 2020). The TaqMan-based real-time PCR provides a multiplexing ability which allows multiple detection in one run while reducing technical bias (Elnifro *et al.*, 2000). In the duplex qPCR specificity experiments performed in this study, Ct values are similar for an amount of 1 pg of *P. olseni* and *P. chesapeaki* gDNA when using corresponding primers and probes in their respective wavelengths (*P. olseni*-Ct value: 26.4 ± 0.07; *P. chesapeaki*-Ct value: 26.7 ± 0.09). Hence, our results support the hypothesis that *P. olseni* and *P. chesapeaki* might possess very similar numbers of ITS2 copies in their genomes allowing an easy comparison of infection intensity between both parasitic species.

In 2020, Marquis *et al.* highlighted the dynamics of *P. marinus*, *P. chesapeaki* and *Haplosporidium nelsoni* in oysters from the Gulf of Maine during summer-fall 2016 and 2017 showing the rising interest of this technique in the management of marine disease (Marquis *et al.*, 2020). Development of a molecular multiplexing approach may be particularly useful in environments where multi-species infection seems to be the rule (Bass *et al.*, 2019). The duplex qPCR method elaborated in this study demonstrates low inter-plate qPCR variation (CV% from 5% to 8 %, Table 2), a testament to its repeatability. Thus, we confirm the utility of TaqMan methodology for the relative quantification of *P. olseni* and *P. chesapeaki* based on reliable and repeatable plasmid-

611 standards (from $2.5x10^1$ to $2.5x10^6$ total copies) and for perkinsosis in Europe generally,
612 which may sometimes involve cryptic dual infections.
613
614 **4.3. PCR inhibitors contained in biological tissue are a very common issue**
615 **linked to qPCR assays**
616 Inhibition effects are recognized issues for parasite detection within host samples
617 (Audemard *et al.*, 2004, 2006). Organic and inorganic compounds present in the host
618 matrix and extracted with gDNA may compromise the efficiency of DNA polymerases
619 and ultimately produce false negative results or erroneous Ct values (De Faveri *et al.*,
620 2009). By producing standard curves supplemented with different concentrations of
621 extract of organs gDNA, we showed here that PCR inhibitors are present in some clam
622 organs but can be resolved by adjusting template gDNA concentrations.
623 In this study, quantification of plasmid standards (a useful and accurate proxy for
624 *Perkinsus*) was tested with six different concentrations of gDNA organs (gills, digestive
625 gland, foot, adductor muscle, mantle and the remaining tissue). For both *Perkinsus*
626 species, gill and digestive gland gDNA extracts did not contain inhibitory compounds in
627 the range of the qPCR detection as already described for gill tissue by Umeda and
628 Yoshinaga (2012). Thus, for these two types of tissue, the gDNA concentration may
629 easily reach 20 $ng.\mu L^{-1}$ within the qPCR framework described above to optimise
630 *Perkinsus* detection and avoid qPCR saturation. Conversely, adductor muscle and the
631 foot on one hand and mantle and remaining tissues on the other showed optimal
632 amplification at host gDNA concentrations of 5 $ng.\mu L^{-1}$ and 2 $ng.\mu L^{-1}$ respectively. These
633 results are surprising because unlike in clams, mantle and the rectum tissue from
634 oysters had no inhibitory effects on qPCR reactions (De Faveri *et al.*, 2009). A lower load
635 of organ gDNA , e.g. 2 $ng.\mu L^{-1}$, decreases the detection limit of the parasite compared to a
636 higher load. Thus, for organs like mantle or remaining tissues, the prevalence of
637 *Perkinsus* parasite may be underestimated in cases of low infection intensity. The
638 thresholds mentioned in this study are basic recommendations for duplex TaqMan-
639 based real-time PCR applied to further studies on the parasite dynamics in the Manila
640 clam. These values are specific to the Manila clam and should be adjusted for each
641 biological model and targeted organ. In spite of inter-individual variability between the
642 three non-infected reference Manila clams used for the inhibitory test, a very clear
643 inhibition trend related to the type of clam organ is observed. This kind of observation

testifies to the appropriateness of gill for routine diagnostic for molecular methods including PCR and qPCR, as this organ is not inhibitory and a reasonable target tissue to detect *Perkinsus* infection.

4.4. Correlation between RFTM methodology and molecular tools: toward a consensus method for the *Perkinsus* diagnosis

This study highlights a significant linear relationship of the duplex qPCR method and the RFTM method on gill tissue samples. Even if the Spearman's coefficient is not as high as expected (Ríos *et al.*, 2020), two hypothesis can explain the difference between both measures. First, there is a fundamental difference between both methodologies in terms of analytical effort. Indeed, a small fraction of total gDNA amount is analysed for the molecular method whereas the RFTM allows counting the entire gill in case of low infection intensity. Secondly, in this study, we decided to exploit each gill of an individual separately (see Material & Methods) instead of shredding both parts together and losing some more fragile parasitic cells. The asymmetrical infection of *Perkinsus* sp. of clam gills may induce some mismatches connecting the two methods. This divergence is mostly observed at the lower limits of the qPCR standards, from $2.5x10^1$ to $2.5x10^2$ total copies. For other standard-points (i.e. from $2.5x10^2$ to $2.5x10^6$ total copies), the higher infection intensities enable a more homogeneous distribution of parasites in the gill and therefore better qPCR detection. Most of the positive individuals (32 of 34 paired-positive Manila clams) were localised in the range of $2.5x10^2$ to $2.5x10^6$ total copies leading to good correlation between RFTM and qPCR assay quantification.

In this study, there was no difference in prevalence (74% with 88% of concordance). However, we observed that the mean infection intensity for the RFTM method ($9.04x10^4$ ± $2.24x10^5$ cells. g^{-1} of wet tissue) seemed lower than the qPCR assay ($3.35x10^5$ ± $8.55x10^5$ cell. g^{-1} of wet tissue). These values should be interpreted with caution because of the small number of individuals sampled (n=50). Such difference could be explained by several hypothetical biases. First, the two methods would interact with parasite cell nuclear count differently, the RFTM assay registering all *Perkinsus* hypnospores present as individual cells, but the qPCR on the other hand registering multinucleate schizonts as greater template DNA abundance equating, inaccurately, to higher numbers of individual parasite cells. The presence of multinucleate schizonts would thus have the effect of

676 inflating parasite intensity as perceived by qPCR. Second, and to the contrary, an
677 incomplete transformation of the intra-host population of trophozoites and schizonts
678 into hypnospores could conceivably lead to RFTM results underestimating the overall
679 infection intensity. This underestimation might be accentuated for the lowest infection
680 intensities (e.g. for 8.1 to $6.0x10^2$ cells.g^{-1} of wet tissue in this study). The physiological
681 status of the parasite and incomplete hypnospore formation could have a much greater
682 impact on the estimation of low infection intensities than intensities comprising
683 thousands of cells per gram of tissue. In addition, the infection intensities of the duplex
684 qPCR are indirectly estimated by a mathematical relationship (y=0.6041x+4.3459;
685 adjusted-R^2 = 0.61) relying on the RFTM counting method. Moreover, this linear
686 equation has a positive value of intercept which impacts strongly lowest values of
687 infection.

688 The molecular method, in other words, measures a total amount of DNA copies
689 regardless of the physiological stage of *Perkinsus* cells while the traditional RFTM
690 method allows the counting of trophozoites that are able to transform into hypnospores.
691 Given their respective biases, it is not surprising that qPCR infection intensity values
692 appear to be higher than those of the RFTM assay. The duplex qPCR method nonetheless
693 is a useful alternative to RFTM for application in many situations.

694

695 ### 4.5. Application of the concept: *in situ* co-infection

696 The relationship between detection methods allowed us to evaluate the prevalence and
697 intensity of infection of both parasites *P. olseni* and *P. chesapeaki* in a same host gill
698 tissue sample. At the Lanton station, co-infection was observed at very low frequency
699 (6%, n=3/50 individuals sampled). *P. olseni* largely dominates the cases of single
700 infection and remains preponderant in co-infections. Conversely, *P. chesapeaki* is less
701 prevalent and present at much lower intensities. We demonstrated that *P. olseni* invades
702 the gill more efficiently, with 95% of occupation compared to *P. chesapeaki*. Indeed, *P.*
703 *chesapeaki* represents only 5% of the parasitic load in the co-infected clams. Initial
704 evidence leads to us believe that the distribution between *P. olseni* and *P. chesapeaki*
705 may not be homogeneous within clam organs. Arzul *et al.* (2012) described in *R.*
706 *decussatus*, via *in situ* hybridisation methodology, that *P. olseni* is more widespread and

abundant in host tissues compared to *P. chesapeaki*. The distribution of *P. olseni* shows a differential organ propagation depending on the infection stage (Wang *et al.*, 2018). Globally, it is assumed that gill infection is representative of the distribution of the parasite through the clam or oyster body (Yarnall *et al*. 2000) but in case of multiple infections the parasite tropism could be different depending on their interactions and their spatial and temporal dynamics (Cui *et al.*, 2018). The development of a duplex qPCR method allowed easier detection and quantification of the co-infection phenomenon and determination of the abundance of each parasite in the infected host. It is critical to consider the whole-body compartment in a host to reach the most accurate diagnostic of perkinsosis caused by *P. olseni* and *P. chesapeaki*.

5. Conclusion

When *P. olseni* and *P. chesapeaki* are detected in a same Manila clam population, a multiplexing qPCR approach is more appropriate even if quantification can be overestimated. RFTM may, conceivably, underestimate results for infection intensity of *Perkinsus,* and we recognize that it is not informative in species-level discrimination when multiple *Perkinsus* species are present. While the standard RFTM culture method and the molecular method are generally equivalent approaches in profiling perkinsosis generically, the specific ability of the qPCR to resolve one *Perkinsus* species or the other will make its use advantageous where species discrimination is necessary. The duplex TaqMan real-time PCR assay is a very sensitive, reproducible and specific method to investigate the *in situ* diversity, distribution and abundance of *Perkinsus* spp. in case of multiple-occurrence. Development of this tool could bring an instantaneous screenshot of the disease state and the dynamics of *Perkinsus* spp. interaction. The localisation and the detection of both parasites are a major challenge in the management of potential valuable hosts living in sympatry. The coupling of both methods is a powerful approach to fully understand perkinsosis dynamics and prevent the spread of these parasites in new hosts and environments.

Acknowledgements

738 SI was funded by a French doctoral research grant from Ecole Doctorale des Sciences de
739 la Mer et du Littoral (EDSML) and Région Bretagne. This work was supported by the
740 ANR project ACHN 2016 PARASED (ANR-16_ACHN_0003) and by the French National
741 program EC2CO (Ecosphère Continentale et côtière) project THRAUSTO (N°13046). The
742 funders had no role in study design, data collection and analysis, decision to publish, or
743 preparation of the manuscript. Samples were performed with Planula 4 vessel (CNRS-
744 INSU, Flotte Océanographique Française).

References

Allam, B., Paillard, C. and Ford, S. E. (2002). Pathogenicity of *Vibrio tapetis*, the aetiological agent of brown ring disease in clams. *Diseases of aquatic organisms* **48**, 221–231. doi: 10.3354/dao048221.

Almeida, M., Berthe, F., Thébault, A. and Dinis, M. T. (1999). Whole clam culture as a quantitative diagnostic procedure of *Perkinsus atlanticus* (Apicomplexa, Perkinsea) in clams *Ruditapes decussatus. Aquaculture* **177**, 325–332. doi:10.1016/S0044-8486(99)00095.

Andrews, J. D. (1996). History of *Perkinsus marinus*, a pathogen of oysters in Chesapeake bay 1950-1984. *Journal of Shellfish Research* **15**, 13–16.

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

Audemard, C., Reece, K. S. and Burreson, E. M. (2004). Real-time PCR for detection and quantification of the protistan parasite *Perkinsus marinus* in environmental waters. *Applied and Environmental Microbiology* **70**, 6611–6618. doi: 10.1128/AEM.70.11.6611-6618.2004.

Audemard, C., Calvo, L. R., Paynter, K. T., Reece, K. S. and 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**, 827–842.

Audemard, C., Carnegie, R. and Burreson, E. (2008). Shellfish tissues evaluated for *Perkinsus* spp. using the Ray's fluid thioglycollate medium culture assay can be

used for downstream molecular assays. *Diseases of Aquatic Organisms* **80**, 235–239. doi: 10.3354/dao01944.

Azevedo, C. (1989). Fine structure of *Perkinsus atlanticus* n. sp. (Apicomplexa, Perkinsea) parasite of the clam *Ruditapes decussatus* from Portugal. *The Journal of Parasitology* **75**, 627. doi: 10.2307/3282915.

Balseiro, P., Montes, J., Conchas, R., Novoa, B. and Figueras, A. (2010). Comparison of diagnostic techniques to detect the clam pathogen *Perkinsus olseni*. *Diseases of Aquatic Organisms* **90**, 143–151. doi: 10.3354/dao02194.

Bass, D., Stentiford, G. D., Wang, H.-C., Koskella, B. and Tyler, C. R. (2019). The pathobiome in animal and plant diseases. *Trends in Ecology & Evolution* **34**, 996–1008. doi: 10.1016/j.tree.2019.07.012.

Batista, F. M., Arzul, I., Pepin, J.-F., Ruano, F., Friedman, C. S., Boudry, P. and Renault, T. (2007). Detection of ostreid herpesvirus 1 DNA by PCR in bivalve molluscs: A critical review. *Journal of Virological Methods* **139**, 1–11. doi: 10.1016/j.jviromet.2006.09.005.

Bertignac, M., Auby, I., Sauriau, P.-G., De Montaudouin, X., Foucard, J. and Martin, S. (2001). Evaluation du stock de palourdes du bassin d'Arcachon, Ifremer Report (Contract report).

Burreson, E. M. and Andrews, J. D. (1988). Unusual intensification of Chesapeake bay oyster diseases during recent drought conditions. *OCEANS '88-A Partnership of Marine Interests. Proceedings* (pp. 799–802) IEEE. doi: 10.1109/OCEANS.1988.794899.

Burreson, E. M., Alvarez, R. S., Martinez, W. and Macedo, L. A. (1994). *Perkinsus-marinus* (Apicomplexa) as a potential source of oyster *Crassostrea virginica*

mortality in coastal lagoons of Tabasco, Mexico. *Diseases of aquatic organisms* **20**, 77. doi: 10.3354/dao020077.

Bushek, D., Ford, S. E. and Allen Jr, S. K. (1994). Evaluation of methods using Ray's fluid thioglycollate medium for diagnosis of *Perkinsus marinus* infection in the eastern oyster, *Crassostrea virginica*. *Annual Review of Fish Diseases* **4**, 201–217. doi: 10.1016/0959-8030(94)90029-9.

Bushek, D., Landau, B. and Scarpa, E. (2008). *Perkinsus chesapeaki* in stout razor clams *Tagelus plebeius* from Delaware bay. *Diseases of Aquatic Organisms* **78**, 243–247. doi: 10.3354/dao01871.

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M. W., Shipley, G. L., Vandesompele, J. and Wittwer, C. T. (2009). The MIQE muidelines: minimum information for publication of quantitative real-time PCR experiments. *Clinical Chemistry* **55**, 611–622. doi: 10.1373/clinchem.2008.112797.

Casas, S. M., La Peyre, J. F., Reece, K. S., Azevedo, C. and Villalba, A. (2002a). Continuous *in vitro* culture of the carpet shell clam *Tapes decussatus* protozoan parasite *Perkinsus atlanticus*. *Diseases of aquatic organisms* **52**, 217–231. doi: 10.3354/dao052217.

Casas, S. M., Villalba, A. and Reece, K. S. (2002b). 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. *Diseases of aquatic organisms* **50**, 51–65. doi:10.3354/dao050051.

Chambouvet, A., Gower, D. J., Jirků, M., Yabsley, M. J., Davis, A. K., Leonard, G., Maguire, F., Doherty-Bone, T. M., Bittencourt-Silva, G. B., Wilkinson, M. and Richards, T. A. (2015). Cryptic infection of a broad taxonomic and geographic

diversity of tadpoles by Perkinsea protists. *Proceedings of the National Academy of Sciences* **112**, E4743–E4751. doi: 10.1073/pnas.1500163112.

Choi, K.-S., Wilson, E. A., Lewis, D. H., Powell, E. N. and Ray, S. M. (1989). The energetic cost of *Perkinsus marinus* parasitism in oysters: quantification of the thioglycollate method. 125–131.

Cold, E. R., Freyria, N. J., Martínez Martínez, J. and Fernández Robledo, J. A. (2016). An agar-based method for plating marine protozoan parasites of the genus *Perkinsus*. *PLOS ONE* **11**, e0155015. doi: 10.1371/journal.pone.0155015.

Coss, C. A., Robledo, J. A., Ruiz, G. M. and Vasta, G. R. (2001). Description of *Perkinsus andrewsi* n. sp. isolated from the Baltic clam (*Macoma balthica*) by characterization of the ribosomal RNA locus, and development of a species-specific PCR-based diagnostic assay. *Journal of Eukaryotic Microbiology* **48**, 52–61.

Cui, Y.-Y., Ye, L.-T., Wu, L. and Wang, J.-Y. (2018). Seasonal occurrence of *Perkinsus* spp. and tissue distribution of *P. olseni* in clam (*Soletellina acuta*) from coastal waters of Wuchuan County, southern China. *Aquaculture* **492**, 300–305. doi: 10.1016/j.aquaculture.2018.04.030.

Da Ros, L. and Canzonier, W. J. (1985). *Perkinsus*, a protistan threat to bivalve culture in the Mediterranean basin. *Bulletin of the European Association of Fish Pathologists* **5**, 23–27.

Dang, C., de Montaudouin, X., Caill-Milly, N. and Trumbiç, Ž. (2010). Spatio-temporal patterns of perkinsosis in the Manila clam *Ruditapes philippinarum* from Arcachon Bay (SW France). *Diseases of Aquatic Organisms* **91**, 151–159. doi: 10.3354/dao02243.

De Faveri, J., Smolowitz, R. M. and Roberts, S. B. (2009). Development and validation of a real-time quantitative PCR assay for the detection and quantification of *Perkinsus marinus* in the eastern oyster, *Crassostrea virginica*. *Journal of Shellfish Research* **28**, 459–465. doi: 10.2983/035.028.0306.

Dhanasekaran, S., Doherty, T. M., Kenneth, J. and Group, T. T. S. (2010). Comparison of different standards for real-time PCR-based absolute quantification. *Journal of immunological methods* **354**, 34–39. doi: 10.1016/j.jim.2010.01.004.

Dittman, D. E., Ford, S. E. and Padilla, D. K. (2001). Effects of *Perkinsus marinus* on reproduction and condition of the eastern oyster, *Crassostrea virginica*, depend on timing. *Journal of Shellfish Research* **20**, 1025–1034.

Duffy, T., Cura, C. I., Ramirez, J. C., Abate, T., Cayo, N. M., Parrado, R., Bello, Z. D., Velazquez, E., Muñoz-Calderon, A., Juiz, N. A., Basile, J., Garcia, L., Riarte, A., Nasser, J. R., Ocampo, S. B., Yadon, Z. E., Torrico, F., de Noya, B. A., Ribeiro, I. and Schijman, A. G. (2013). Analytical performance of a multiplex real-time PCR assay using TaqMan probes for quantification of *Trypanosoma cruzi* satellite DNA in blood samples. *PLoS Neglected Tropical Diseases* **7**, e2000. doi: 10.1371/journal.pntd.0002000.

Elnifro, E. M., Ashshi, A. M., Cooper, R. J. and Klapper, P. E. (2000). Multiplex PCR: optimization and application in diagnostic virology. *Clinical microbiology reviews* **13**, 559–570. doi: 10.1128/CMR.13.4.559.

European Commission - DG for health and food safety (2018). Overview report: animal health controls for bivalve mollusc aquaculture.

Flassch, J.-P. and Leborgne, Y. (1994). Introduction in Europe, from 1972 to 1980, of the Japanese Manila clam (*Tapes philippinarum*) and the effects on aquaculture production and natural settlement. In *Introductions and Transfers of Aquatic*

species. Selected papers from a Symposium Held in Halifax, Nova Scotia, 12-13 June 1990.

Food and Agriculture Organization of the United Nations (2018). FishStatJ - Software for Fishery and Aquaculture Statistical Time Series.

Gauthier, J. D., Miller, C. R. and Wilbur, A. E. (2006). Taqman® mgb real-time PCR approach to quantification of *Perkinsus marinus* and *Perkinsus* spp. in oysters. *Journal of Shellfish Research* **25**, 619–624. doi: 10.2983/0730-8000(2006)25[619:TMRPAT]2.0.CO;2.

Goggin, C. and Lester, R. (1995). *Perkinsus*, a protistan parasite of abalone in Australia: A review. *Marine and Freshwater Research* **46**, 639. doi: 10.1071/MF9950639.

Hamaguchi, M., Suzuki, N., Usuki, H. and Ishioka, H. (1998). *Perkinsus* protozoan infection in short-necked clam *Tapes* (= *Ruditapes*) *philippinarum* in Japan. *Fish pathology* **33**, 473–480. doi: 10.3147/jsfp.33.473.

Hardegen, C., Messler, S., Henrich, B., Pfeffer, K., Würthner, J. and MacKenzie, C. R. (2010). A set of novel multiplex Taqman real-time PCRs for the detection of diarrhoeagenic *Escherichia coli* and its use in determining the prevalence of EPEC and EAEC in a university hospital. *Annals of clinical microbiology and antimicrobials* **9**, 5. doi: 10.1186/1476-0711-9-5.

Harvell, C. D. (2002). Climate warming and disease risks for terrestrial and marine biota. *Science* **296**, 2158–2162. doi: 10.1126/science.1063699.

Hohweyer, J., DumèTre, A., Aubert, D., Azas, N. and Villena, I. (2013). Tools and methods for detecting and characterizing *Giardia*, *Cryptosporidium*, and *Toxoplasma* parasites in marine mollusks. *Journal of Food Protection* **76**, 1649–1657. doi: 10.4315/0362-028X.JFP-13-002.

Itoh, N., Meyer, G., Tabata, A., Lowe, G., Abbott, C. and Johnson, S. (2013). Rediscovery of the Yesso scallop pathogen *Perkinsus qugwadi* in Canada, and development of PCR tests. *Diseases of Aquatic Organisms* **104**, 83–91. doi: 10.3354/dao02578.

Jenkins, C., Hick, P., Gabor, M., Spiers, Z., Fell, S., Gu, X., Read, A., Go, J., Dove, M., O'Connor, W., Kirkland, P. and Frances, J. (2013). Identification and characterisation of an ostreid herpesvirus-1 microvariant (OsHV-1 µ-var) in *Crassostrea gigas* (Pacific oysters) in Australia. *Diseases of Aquatic Organisms* **105**, 109–126. doi: 10.3354/dao02623.

Kuhar, U., Barlič-Maganja, D. and Grom, J. (2013). Development and validation of TaqMan probe based real time PCR assays for the specific detection of genotype A and B small ruminant lentivirus strains. *BMC veterinary research* **9**, 172. doi: 10.1186/1746-6148-9-172.

Langton, S. D., Chevennement, R., Nagelkerke, N. and Lombard, B. (2002). Analysing collaborative trials for qualitative microbiological methods: accordance and concordance. *International Journal of Food Microbiology* **79**, 175–181. doi: 10.1016/S0168-1605(02)00107-1.

Lee, M.-K., Cho, B.-Y., Lee, S.-J., Kang, J.-Y., Jeong, H. D., Huh, S. H. and Huh, M.-D. (2001). Histopathological lesions of Manila clam, *Tapes philippinarum*, from Hadong and Namhae coastal areas of Korea. *Aquaculture* **201**, 199–209. doi: 10.1016/S0044-8486(01)00648-2.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, Buchner, A., Lai, T., Steppi, S., Jobb, G., Förster, W., Brettske, I., Gerber, S., Ginhart, A. W., Gross, O., Grumann, S., Hermann, S., Jost, R., König, A., Liss, T., Lüßmann, R., May, M., Nonhoff, B., Reichel, B., Strehlow, R., Stamatakis, A., Stuckmann,

N., Vilbig, A., Lenke, M., Ludwig, T., Bode, A. and Schleifer, K. (2004). ARB: a software environment for sequence data. *Nucleic Acids Research* **32**, 1363–1371. doi: 10.1093/nar/gkh293.

Mackin, J. G. (1951). Histopathology of infection of *Crassostrea virginica* (Gmelin) by *Dermocystidium marinum* Mackin, Owen, and Collier. *Bulletin of Marine Science* **1**, 72–87.

Mackin, J. G., Owen, H. M. and Collier, A. (1950). Preliminary note on the occurrence of a new protistan parasite, *Dermocystidium marinum* n. sp. in *Crassostrea virginica* (Gmelin). *Science* **111**, 328. doi: 10.1126/science.111.2883.328.

Marquis, N. D., Bishop, T. J., Record, N. R., Countway, P. D. and Fernández Robledo, J. A. (2020). A qPCR-based survey of *Haplosporidium nelsoni* and *Perkinsus* spp. in the Eastern oyster, *Crassostrea virginica* in Maine, USA. *Pathogens* **9**, 256. doi: 10.3390/pathogens9040256.

Montes, J., Durfort, M. and García-Valero, J. (2001). Parasitism by the protozoan *Perkinsus atlanticus* favours the development of opportunistic infections. *Diseases of Aquatic Organisms* **46**, 57–66. doi: 10.3354/dao046057.

Nam, K.-W., Jeung, H.-D., Song, J.-H., Park, K.-H., Choi, K.-S. and Park, K.-I. (2018). High parasite burden increases the surfacing and mortality of the Manila clam (*Ruditapes philippinarum*) in intertidal sandy mudflats on the west coast of Korea during hot summer. *Parasites & Vectors* **11**. doi: 10.1186/s13071-018-2620-3.

Novoa, B., Ordás, M. C. and Figueras, A. (2002). Hypnospores detected by RFTM in clam (*Ruditapes decussatus*) tissues belong to two different protozoan organisms, *Perkinsus atlanticus* and a *Perkinsus*-like organism. *Aquaculture* **209**, 11–18. doi: 10.1016/S0044-8486(01)00803-1.

OIE (2019a). Aquatic Animal Health Code (2019).

OIE (2019b). Chapter 2.4.7. - Infection with Perkinsus olseni. *Manual of Diagnostic Tests for Aquatic Animals*.

Pagenkopp Lohan, K. M., Hill-Spanik, K. M., Torchin, M. E., Fleischer, R. C., Carnegie, R. B., Reece, K. S. and Ruiz, G. M. (2018). Phylogeography and connectivity of molluscan parasites: *Perkinsus* spp. in Panama and beyond. *International Journal for Parasitology* **48**, 135–144. doi: 10.1016/j.ijpara.2017.08.014.

Park, K.-I. and Choi, K.-S. (2001). Spatial distribution of the protozoan parasite *Perkinsus* sp. found in the Manila clams, *Ruditapes philippinarum*, in Korea. *Aquaculture* **203**, 9–22. doi: 10.1016/S0044-8486(01)00619-6.

Pretto, T., Zambon, M., Civettini, M., Caburlotto, G., Boffo, L., Rossetti, E. and Arcangeli, G. (2014). Massive mortality in Manila clams (*Ruditapes philippinarum)* farmed in the Lagoon of Venice, caused by *Perkinsus olseni*. *Bulletin- European Association of Fish Pathologists* **34**, 43–53.

Putaporntip, C., Hongsrimuang, T., Seethamchai, S., Kobasa, T., Limkittikul, K., Cui, L. and Jongwutiwes, S. (2009). Differential prevalence of *Plasmodium* infections and cryptic *Plasmodium knowlesi* malaria in humans in Thailand. *The Journal of Infectious Diseases* **199**, 1143–1150. doi: 10.1086/597414.

Ramilo, A., Pintado, J., Villalba, A. and Abollo, E. (2016). *Perkinsus olseni* and *P. chesapeaki* detected in a survey of perkinsosis of various clam species in Galicia (NW Spain) using PCR–DGGE as a screening tool. *Journal of invertebrate pathology* **133**, 50–58. doi: 10.1016/j.jip.2015.11.012.

Ray, S. M. (1952). A culture technique for the diagnosis of infections with *Dermocystidium marinum* (Mackin, Owen, and Collier) in oysters. *Science* **116**, 360. doi: 10.1126/science.116.3014.360.

Reece, K., Dungan, C. and Burreson, E. (2008). Molecular epizootiology of *Perkinsus marinus* and *P. chesapeaki* infections among wild oysters and clams in Chesapeake bay, USA. *Diseases of Aquatic Organisms* **82**, 237–248. doi: 10.3354/dao01997.

Renault, T., Le Deuff, R.-M., Lipart, C. and Delsert, C. (2000). Development of a PCR procedure for the detection of a herpes-like virus infecting oysters in France. *Journal of Virological Methods* **88**, 41–50. doi: 10.1016/S0166-0934(00)00175-0.

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

Ruano, F. and Cachola, R. (1986). Outbreak of a severe epizootic of *Perkinsus marinus* (Levin-78) at Ria de Faro clam's culture beds. In *Proceedings of 2nd International Colloque Pathology Marine Aquatic*, pp. 4–42.

Shamal, P., Zacharia, P. U., Binesh, C. P., Pranav, P., Suja, G., Asokan, P. K., Pradeep, M. A., Rithesh, R., Vijayan, K. K. and Sanil, N. K. (2018). *Perkinsus olseni* in the short neck yellow clam, *Paphia malabarica* (Chemnitz, 1782) from the southwest coast of India. *Journal of Invertebrate Pathology* **159**, 113–120. doi: 10.1016/j.jip.2018.10.001.

Takahashi, M., Yoshinaga, T., Waki, T., Shimokawa, J. and Ogawa, K. (2009). Development of a PCR-RFLP method for differentiation of *Perkinsus olseni* and *P.*

honshuensis in the Manila clam *Ruditapes philippinarum*. *Fish Pathology* **44**, 185–188. doi: 10.3147/jsfp.44.185.

Tall, B. D., La Peyre, J. F., Bier, J. W., Miliotis, M. D., Hanes, D. E., Kothary, M. H., Shah, D. B. and Faisal, M. (1999). *Perkinsus marinus* extracellular protease modulates survival of *Vibrio vulnificus* in eastern oyster (*Crassostrea virginica*) hemocytes. *Applied and Environmental Microbiology* **65**, 4261–4263. doi: 10.1128/AEM.65.9.4261-4263.1999.

Ulrich, P. N., Ewart, J. W. and Marsh, A. G. (2007). Prevalence of *Perkinsus marinus* (Dermo), *Haplosporidium nelsoni* (MSX), and QPX in bivalves of Delaware's inland bays and quantitative, high-throughput diagnosis of Dermo by qPCR. *The Journal of Eukaryotic Microbiology*, doi: 10.1111/j.1550-7408.2007.00293.x.

Umeda, K. and Yoshinaga, T. (2012). Development of real-time PCR assays for discrimination and quantification of two *Perkinsus* spp. in the Manila clam *Ruditapes philippinarum*. *Diseases of Aquatic Organisms* **99**, 215–225. doi: 10.3354/dao02476.

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B. C., Remm, M. and Rozen, S. G. (2012). Primer3—new capabilities and interfaces. *Nucleic Acids Research* **40**, e115–e115. doi: 10.1093/nar/gks596.

Wang, Y., Yoshinaga, T. and Itoh, N. (2018). New insights into the entrance of *Perkinsus olseni* in the Manila clam, *Ruditapes philippinarum*. *Journal of Invertebrate Pathology* **153**, 117–121. doi: 10.1016/j.jip.2018.03.005.

Ward, J. R. and Lafferty, K. D. (2004). The elusive baseline of marine disease: are diseases in ocean ecosystems increasing? *PLoS biology* **2**. doi: 10.1371/journal.pbio.0020120.

Winnepenninckx, B., Backeljau, T. and De Wachter, R. (1993). Extraction of high molecular weight DNA from molluscs. *Trends in Genetics* **9**, 407.

Xie, Z., Xie, L., Fan, Q., Pang, Y., Deng, X., Xie, Z. Q., Liu, J. and Khan, M. I. (2013). A duplex quantitative real-time PCR assay for the detection of *Haplosporidium* and *Perkinsus* species in shellfish. *Parasitology Research* **112**, 1597–1606. doi: 10.1007/s00436-013-3315-5.

Yarnall, H. A., Reece, K. S., Stokes, N. A. and Burreson, E. M. (2000). A quantitative competitive polymerase chain reaction assay for the oyster pathogen *Perkinsus marinus*. *Journal of Parasitology* **86**, 827–837. doi: 10.1645/0022-3395(2000)086[0827:AQCPCR]2.0.CO;2.

Figure 1. Schematic position of primers and probes designed for duplex TaqMan PCR within the ITS1-5.8S-ITS2 sequence region of ribosomal genes of *Perkinsus* spp.

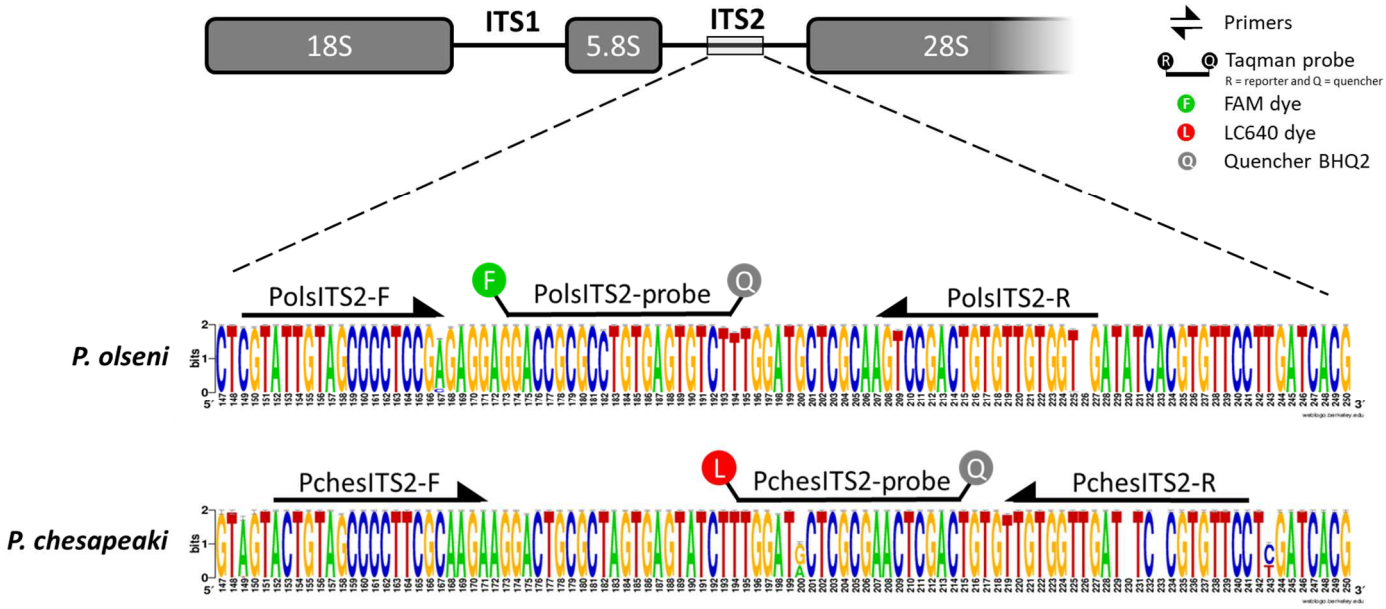


Figure 2. Specificity of primers and probes designed for the duplex qPCR assay. Primers and probes specificity were tested by conducting duplex real-time PCR on *P. olseni* gDNA (1 pg), *P. chesapeaki* gDNA (1 pg), *R. philippinarum* gills gDNA (50 ng) and pure water. Positive amplification is significant when the fluorescence values exceed the threshold indicated by the horizontal red line. Amplification curves demonstrating the specificity of *P. olseni* primers on *P. olseni* gDNA between 465-510 nm and the specificity of *P. chesapeaki* set on *P. chesapeaki* gDNA between 618-660 nm. NTC: Non-Template Control (water).

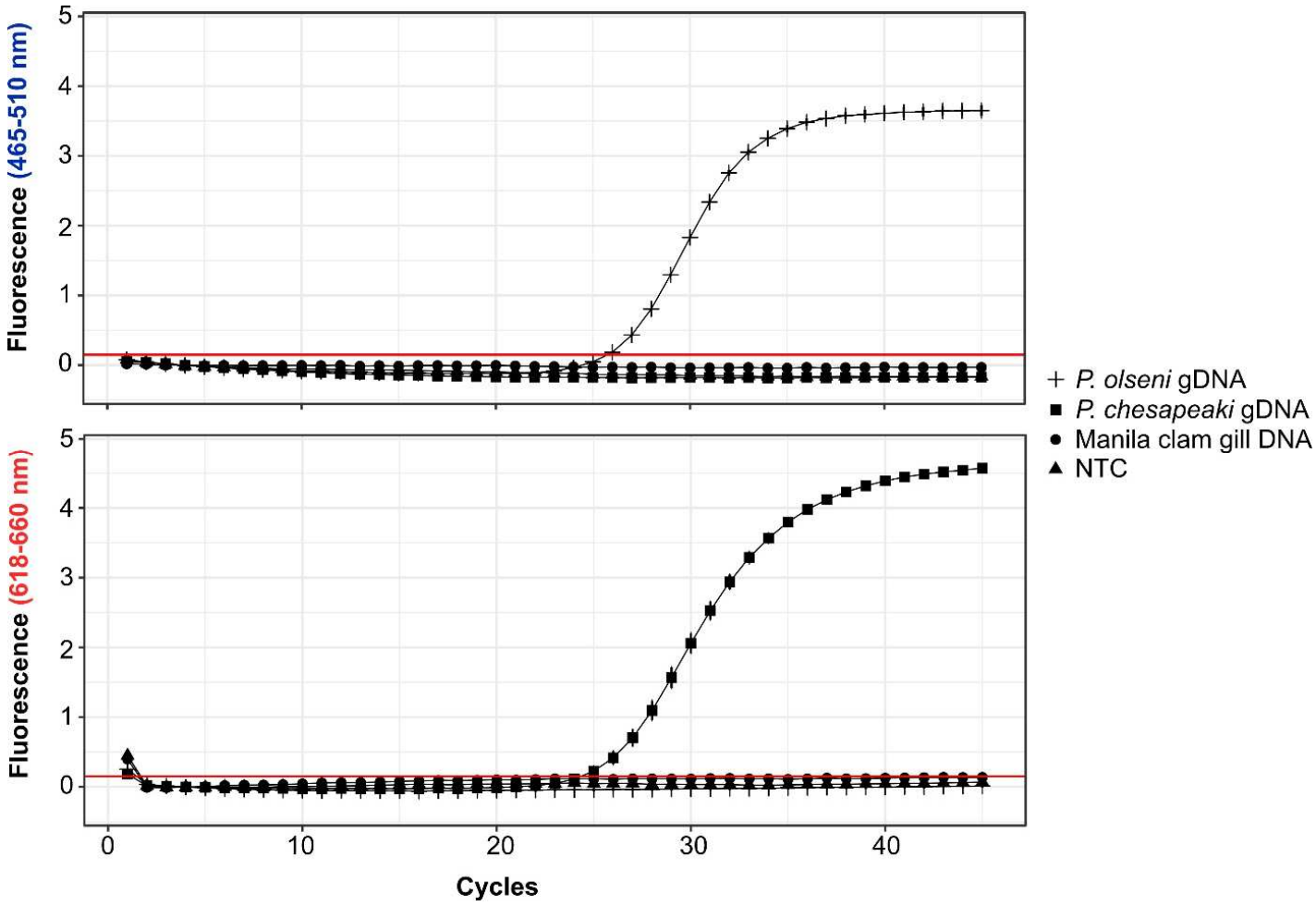


Figure 3. Standards and efficiency evaluation of real-time PCR assays. Standard curves based on *P. olseni*-plasmids and on *P. chesapeaki*-plasmids serial dilution were determined for downstream analysis. *P. olseni* standard relationship: y=-3.33x+39.48; *P. chesapeaki* standard relationship: y=-3.38x+40.30 Ct: Cycle threshold value. E: Efficiency (E) of the qPCR assay is calculated as follow: $E = (10^{(-1/slope)} - 1) \times 100\%$.

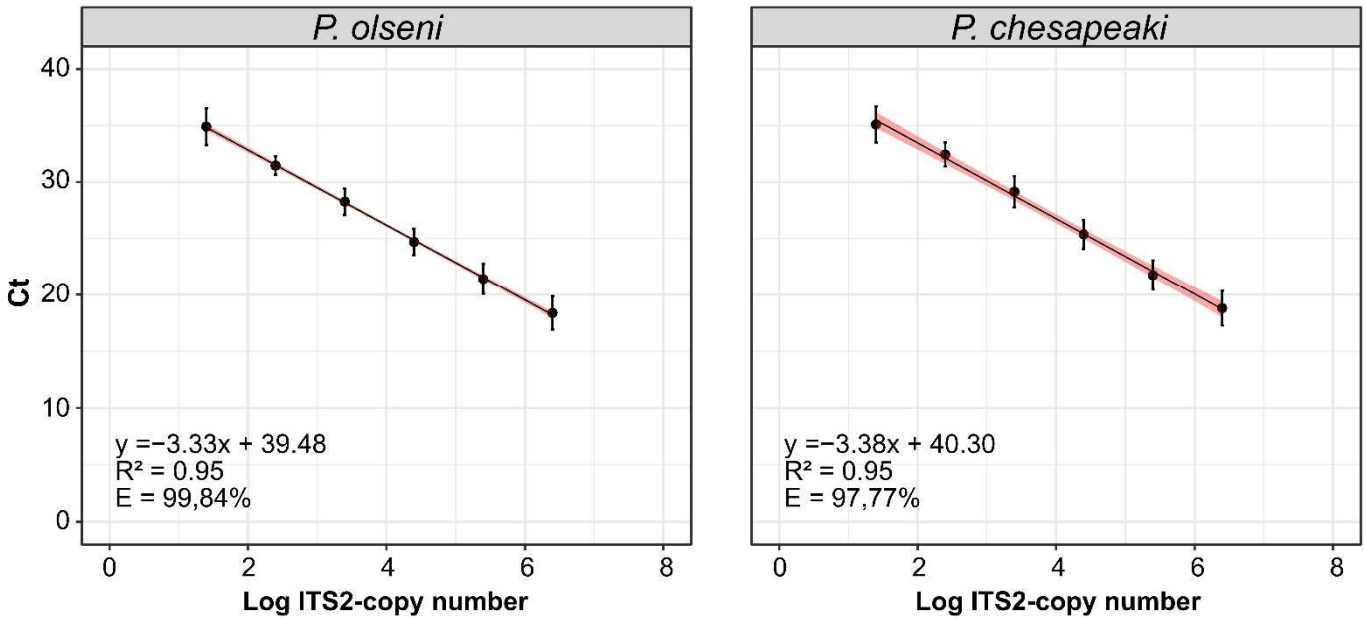


Figure 4. Sensitivity and inhibitory effects induced by four concentrations of gDNA from the six different types of tissue samples (gill, digestive gland, adductor muscle, foot, mantle and remaining tissue). The sensitivity of the real-time duplex PCR is represented for six (A) *P. olseni*-plasmids and (B) *P. chesapeaki*-plasmids dilution combined with four gDNA concentrations: 2 ng.µl^{-1}, 5 ng.µl^{-1}, 10 ng.µl^{-1} and 20 ng.µl^{-1}. The specific standard curve is represented by the red curve with round red circles. Ct: Cycle threshold value.

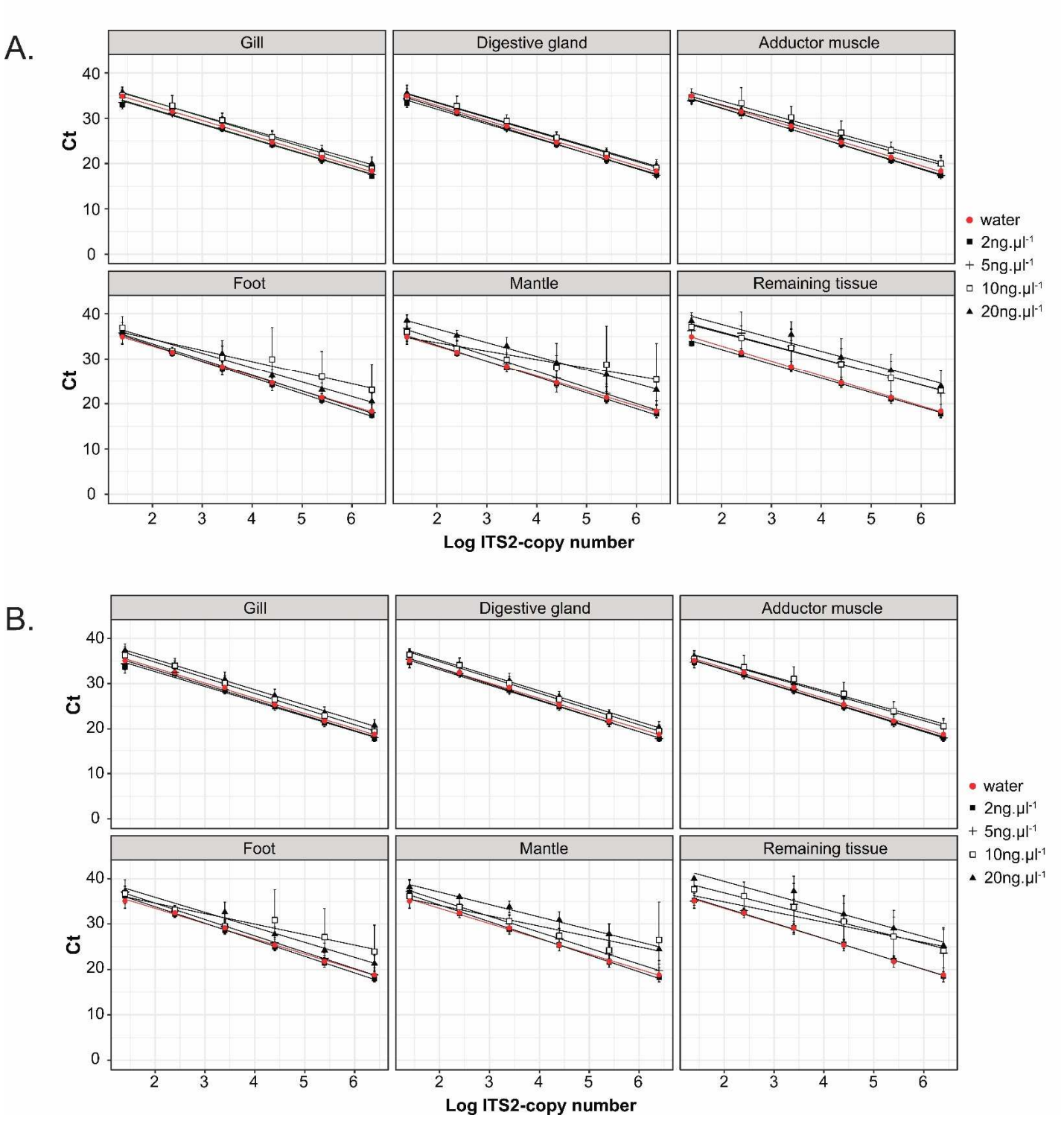


Figure 5. Linear regression between infection intensities determined by RFTM assay (nb. of cells.g of wet gill^{-1}) and by qPCR assay (nb. of copies.g of wet gill^{-1}). The infection intensities determined by RFTM are based on the global counting of *Perkinsus* sp. hypnospores. The infection intensities determined by duplex qPCR are based on the sum of *P. olseni* and *P. chesapeaki* copies. RFTM and qPCR relationship: y=-0.60x+4.36. Ct: Cycle threshold value. r: Pearson's coefficient.

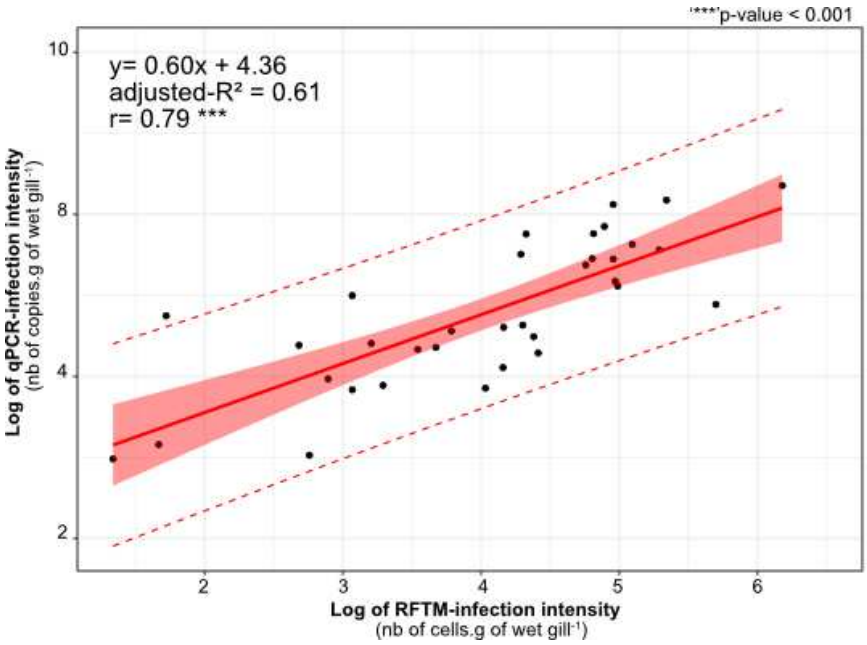


Figure S1. Map of the sampling site in Arcachon Bay (SW France, Atlantic coast, 44°41'60" N;1°10' W) indicating the collecting point at Lanton.

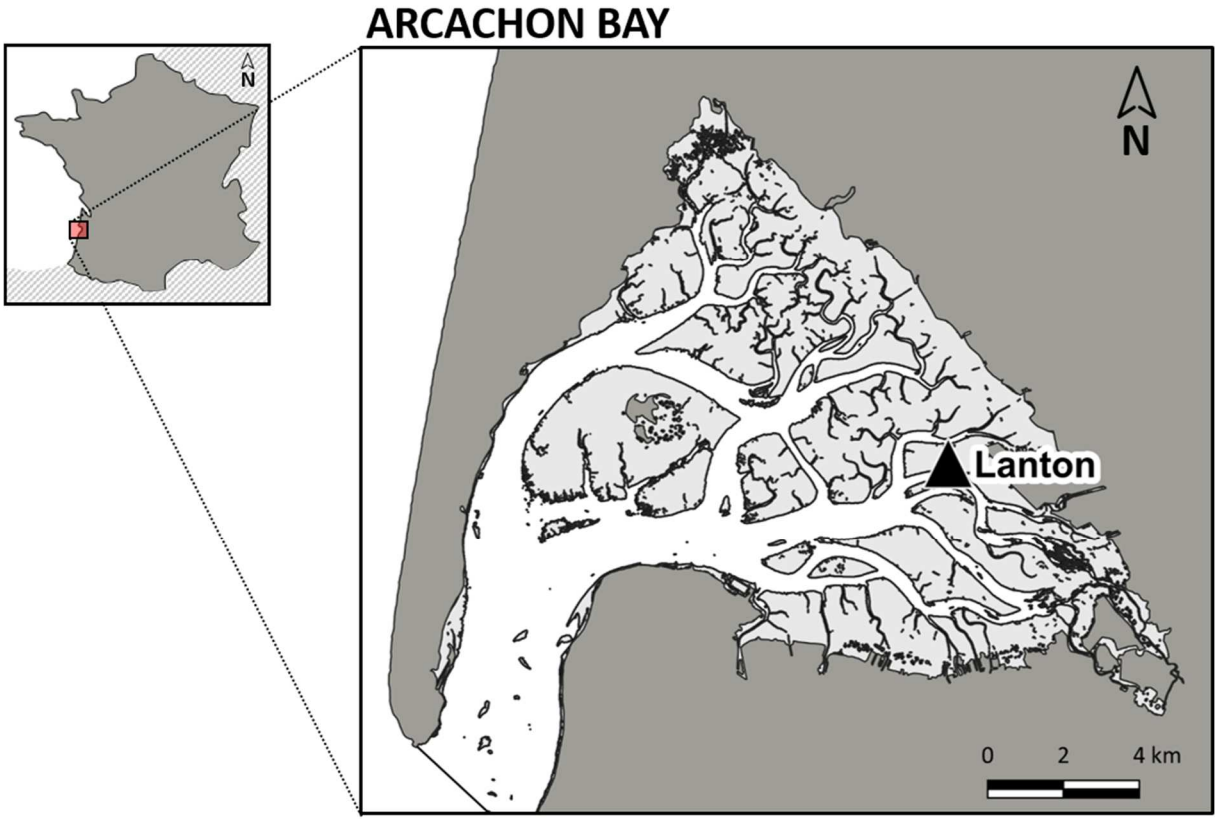


Figure S2. Neighbour joining (NJ) tree of *Perkinsus* genus diversity using the ITS1-5.8S-ITS2 region of the ribosomal operon. The phylogeny was calculated from 57 taxa and 633 characters alignment position. NJ distances (1000 replicates), Maximum Likelihood (ML) bootstraps (1000 replicates) and Bayesian posterior probability (2,000,000 generations, HKY+G model) were added at each node as follows: black circle: bootstrap values are equal to or higher than 80%/80%/0.8; white circle: bootstrap values are higher than 60%/60%/0.6; '+': bootstrap value is below 60% or 600 and if the topology is consistent; '-': the topology is not consistent. The double-slides means the branch has been reduced by two. Five sequences of *P. qugwadi* were used as an outgroup.

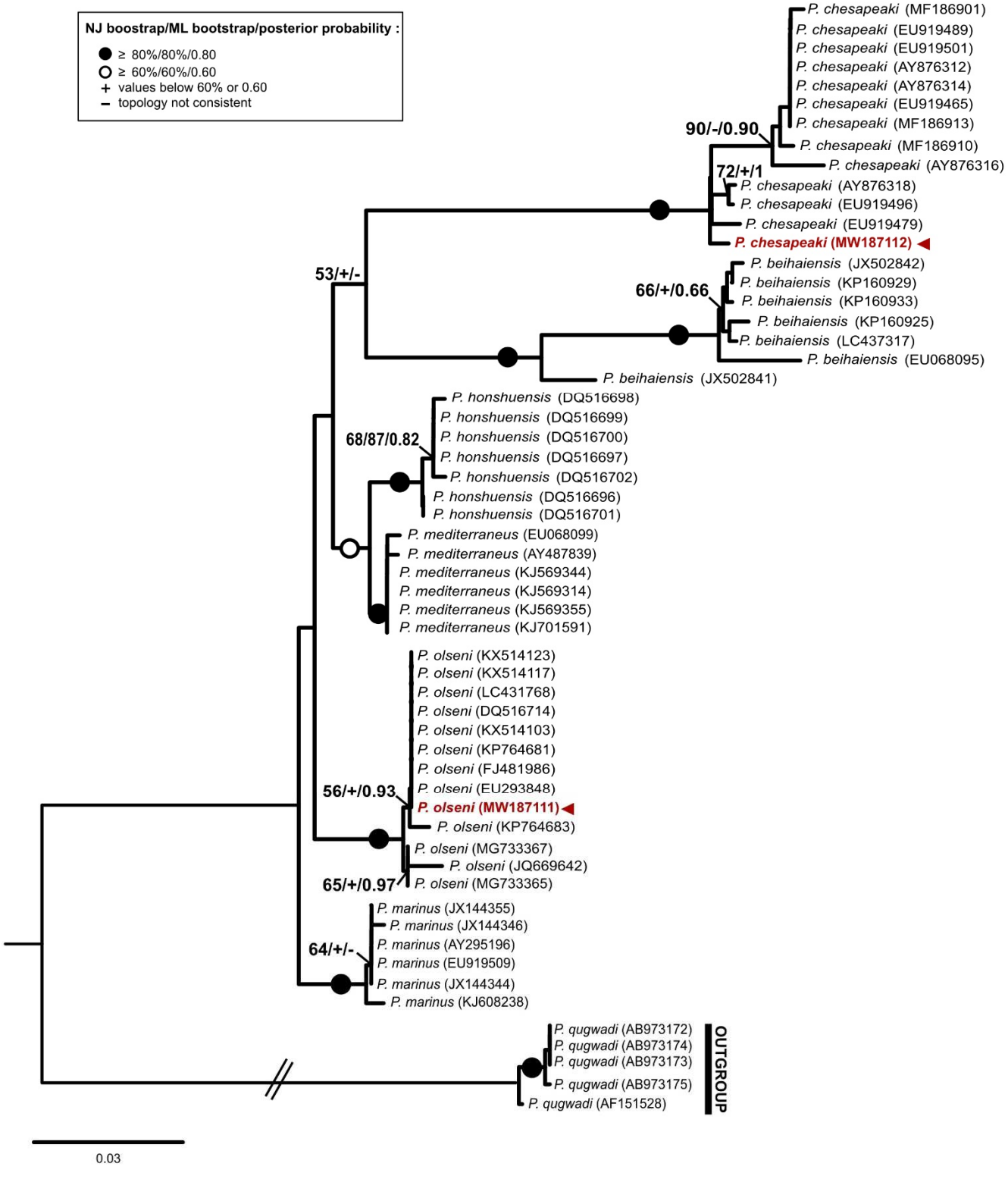


Table 1. Primer and probe sequences specific to *P. olseni* and *P. chesapeaki* ITS2 sequence region of nuclear ribosomal DNA. Excitation wavelengths of FAM fluorochrome is 465-510 nm and of LC640 fluorochrome is 618-660 nm.

Target	Primer /probe	Sequence (5'->3')	Tm (°C)	Amplicon size
ITS 2 *P. olseni*	PolsITS2-F	CACCACAACACAGTCGGAC	58,8	
	PolsITS2-R	CGTATTGTAGCCCCTCCGA	58,8	76 bp
	PolsITS2-probe	**FAM**-GACACTCACAGGCGCGGTCC-**[BHQ2]**	65,7	
ITS 2 *P. chesapeaki*	PchesITS2-F	GGAACACGGAATCAACCACA	57,3	
	PchesITS2-R	ACTGTAGCCCCTTCGCAAG	58,8	87 bp
	PchesITS2-probe	**LC640**-AGTCGAGTTCGCGAGCATCCAA-**[BHQ2]**	63,3	

Table 2. Repeatability of the duplex qPCR assay estimated by the coefficient of variation (CV; %) for *P. olseni* and *P. chesapeaki* plasmid standard concentrations (from 2.5×10^1 to 2.5×10^6 copies).

	number of copies					
	2.5×10^1	2.5×10^2	2.5×10^3	2.5×10^4	2.5×10^5	2.5×10^6
P. olseni standard	5 %	3 %	4 %	5 %	6 %	8 %
P. chesapeaki standard	5 %	3 %	5 %	5 %	6 %	8 %

Table 3. Comparison of Perkinsosis prevalences on gill tissue samples at Lanton determined by the standard RFTM methodology and the duplex qPCR methodology. Unq.: unquantifiable.

Type of method	nb. of clams tested	nb. of infected clams	nb. of healthy clams	**Prevalence (%)**				
				Perkinsus sp.	*P. olseni*	*P. chesapeaki*	Single-infection	Co-infection
RFTM	50	37	13	74	Unq.	Unq.	Unq.	Unq.
duplex qPCR	50	37	13	74	74	6	68	6

Table 4. Concordance parameters of duplex qPCR method and RFTM method. The a, b, c and d are expressed as number of individuals based on a total of 50 clams (n).

		RFTM assay	
		positive	negative
Duplex qPCR assay	positive	34 **(a)**	3 **(b)**
	negative	3 **(c)**	10 **(d)**

Concordance	a+d/n	88 %
Discordance	b+c/n	12 %

Table S1. Characteristic of the Perkinsosis at Lanton station. 'n', is the number of Manila clams sampled in each station. Prevalence is estimated using the RFTM/NaOH digestion methodology (Choi *et al.*, 1989) and represents the number of infected clams on the total of clams sampled (%). The infection intensity is calculated by the mean of the number of hypnospores per gram of wet gill weight (g) in infected hosts. SD, is the standard-deviation. nd: not determined.

Site	Latitude (X)	Longitude (Y)	Type of analysis	n	Prevalence RFTM (%)	Mean infection intensity (nb. of cells.g^{-1} of wet tissue ± SD)
Lanton	44°41'31"N	1°4'48"W	Perkinsosis diagnostis	50	74	$9.07x10^4 \pm 2.24x10^5$
			Culture	5	100	nd

Table S2. GenBank ITS1-5.8S-ITS2 sequence region belonging to *Perkinsus* genus used in phylogenetic analysis to design and test *P. olseni* and *P. chesapeaki* specific primers and probes in duplex qPCR assays.

Accession no.	Class	Order	Genus	Species	Strain/Clone	Reference
JX144346	Perkinsea	Perkinsida	*Perkinsus*	*marinus*	60_2	(da Silva *et al.*, 2013)
KJ608238	Perkinsea	Perkinsida	*Perkinsus*	*marinus*	70_3_C_c5	(da Silva *et al.*, 2014)
JX144355	Perkinsea	Perkinsida	*Perkinsus*	*marinus*	69	(da Silva *et al.*, 2013)
EU919509	Perkinsea	Perkinsida	*Perkinsus*	*marinus*	PXBICv25-B9-C5	(Reece *et al.*, 2008)
AY295196	Perkinsea	Perkinsida	*Perkinsus*	*marinus*	SC2_4_7	(Brown *et al.*, 2004)
JX144344	Perkinsea	Perkinsida	*Perkinsus*	*marinus*	60	(da Silva *et al.*, 2013)
KJ569344	Perkinsea	Perkinsida	*Perkinsus*	*mediterraneus*	H08_3_O6	(Valencia *et al.*, 2014)
AY487839	Perkinsea	Perkinsida	*Perkinsus*	*mediterraneus*	D10	(Casas *et al.*, 2004)
KJ569314	Perkinsea	Perkinsida	*Perkinsus*	*mediterraneus*	H03_3_O4	(Valencia *et al.*, 2014)
KJ569355	Perkinsea	Perkinsida	*Perkinsus*	*mediterraneus*	H03_28_A3	(Valencia *et al.*, 2014)
EU068099	Perkinsea	Perkinsida	*Perkinsus*	*mediterraneus*	Jer2_50	(Casas *et al.*, 2008)
KJ701591	Perkinsea	Perkinsida	*Perkinsus*	*mediterraneus*	12	(Ramilo *et al.*, 2015)
DQ516701	Perkinsea	Perkinsida	*Perkinsus*	*honshuensis*	Mie3gH8_3	(Dungan and Reece, 2006)
DQ516696	Perkinsea	Perkinsida	*Perkinsus*	*honshuensis*	Mie3g_2	(Dungan and Reece, 2006)
DQ516702	Perkinsea	Perkinsida	*Perkinsus*	*honshuensis*	Mie3gH8_4	(Dungan and Reece, 2006)
DQ516699	Perkinsea	Perkinsida	*Perkinsus*	*honshuensis*	Mie3gH8_1	(Dungan and Reece, 2006)
DQ516700	Perkinsea	Perkinsida	*Perkinsus*	*honshuensis*	Mie3gH8_2	(Dungan and Reece, 2006)
DQ516698	Perkinsea	Perkinsida	*Perkinsus*	*honshuensis*	Mie3g_4	(Dungan and Reece, 2006)
DQ516697	Perkinsea	Perkinsida	*Perkinsus*	*honshuensis*	Mie3g_3	(Dungan and Reece, 2006)
JX502841	Perkinsea	Perkinsida	*Perkinsus*	*beihaiensis*	138	(Sabry *et al.*, 2013)
KP160933	Perkinsea	Perkinsida	*Perkinsus*	*beihaiensis*	CBMA287	(Queiroga *et al.*, 2015)
LC437317	Perkinsea	Perkinsida	*Perkinsus*	*beihaiensis*	Koitogawa_4	Itoh et al. 2019 unpublished
KP160925	Perkinsea	Perkinsida	*Perkinsus*	*beihaiensis*	CBMA92	(Queiroga *et al.*, 2015)
KP160929	Perkinsea	Perkinsida	*Perkinsus*	*beihaiensis*	CBMA142	(Queiroga *et al.*, 2015)
JX502842	Perkinsea	Perkinsida	*Perkinsus*	*beihaiensis*	34	(Sabry *et al.*, 2013)
EU068095	Perkinsea	Perkinsida	*Perkinsus*	*beihaiensis*	QZ0649	(Moss *et al.*, 2008)
AB973173	Perkinsea	Perkinsida	*Perkinsus*	*qugwadi*	8302-1	(Itoh *et al.*, 2013)
AB973174	Perkinsea	Perkinsida	*Perkinsus*	*qugwadi*	8302-11	(Itoh *et al.*, 2013)
AB973175	Perkinsea	Perkinsida	*Perkinsus*	*qugwadi*	8302-51	(Itoh *et al.*, 2013)
AB973172	Perkinsea	Perkinsida	*Perkinsus*	*qugwadi*	6675-2	(Itoh *et al.*, 2013)
AF151528	Perkinsea	Perkinsida	*Perkinsus*	*qugwadi*	-	Hervio et al. 1999 unpublished
EU919479	Perkinsea	Perkinsida	*Perkinsus*	*chesapeaki*	EBNPMb2-B5-D12	(Reece *et al.*, 2008)
EU919489	Perkinsea	Perkinsida	*Perkinsus*	*chesapeaki*	PXBIMa10-D10-E4	(Reece *et al.*, 2008)
EU919501	Perkinsea	Perkinsida	*Perkinsus*	*chesapeaki*	YRKCMb1-G2-G8	(Reece *et al.*, 2008)
AY876312	Perkinsea	Perkinsida	*Perkinsus*	*chesapeaki*	ATCC 50807	(Burreson *et al.*, 2005)
AY876314	Perkinsea	Perkinsida	*Perkinsus*	*chesapeaki*	ATCC 50807	(Burreson *et al.*, 2005)

EU919465	Perkinsea	Perkinsida	*Perkinsus*	*chesapeaki*	CRBSTp9	(Reece *et al.*, 2008)
EU919496	Perkinsea	Perkinsida	*Perkinsus*	*chesapeaki*	PXSATp6-A7-A8	(Reece *et al.*, 2008)
AY876318	Perkinsea	Perkinsida	*Perkinsus*	*chesapeaki*	ATCC PRA-65	(Burreson *et al.*, 2005)
AY876316	Perkinsea	Perkinsida	*Perkinsus*	*chesapeaki*	ATCC PRA-65	(Burreson *et al.*, 2005)
MF186901	Perkinsea	Perkinsida	*Perkinsus*	*chesapeaki*	A9-1	(Reece *et al.*, 2017)
MF186913	Perkinsea	Perkinsida	*Perkinsus*	*chesapeaki*	A5_4	(Reece *et al.*, 2017)
MF186910	Perkinsea	Perkinsida	*Perkinsus*	*chesapeaki*	A7_2	(Reece *et al.*, 2017)
JQ669642	Perkinsea	Perkinsida	*Perkinsus*	*olseni*	05067_3P2/1_6	(Arzul *et al.*, 2012)
KX514117	Perkinsea	Perkinsida	*Perkinsus*	*olseni*	MS2-2	Cho et al. 2018 unpublished
KX514123	Perkinsea	Perkinsida	*Perkinsus*	*olseni*	SS2-2	Cho et al. 2018 unpublished
LC431768	Perkinsea	Perkinsida	*Perkinsus*	*olseni*	shoG	Imajoh et al. 2018 unpublished
DQ516714	Perkinsea	Perkinsida	*Perkinsus*	*olseni*	Mie13v_8	(Dungan and Reece, 2006)
MG733367	Perkinsea	Perkinsida	*Perkinsus*	*olseni*	PM51DH1	(Shamal *et al.*, 2018)
MG733365	Perkinsea	Perkinsida	*Perkinsus*	*olseni*	PM45EL4	(Shamal *et al.*, 2018)
KP764683	Perkinsea	Perkinsida	*Perkinsus*	*olseni*	8	(Ramilo *et al.*, 2016)
KX514103	Perkinsea	Perkinsida	*Perkinsus*	*olseni*	WD4-2	Cho et al. 2018 unpublished
KP764681	Perkinsea	Perkinsida	*Perkinsus*	*olseni*	6	(Ramilo *et al.*, 2016)
FJ481986	Perkinsea	Perkinsida	*Perkinsus*	*olseni*	Rp1	Elandaloussi et al., 2008 unpublished
EU293848	Perkinsea	Perkinsida	*Perkinsus*	*olseni*	-	(Elandaloussi *et al.*, 2009)

Table S3. *In silico* specificity of primers using Primer-BLAST (Ye *et al.*, 2012). Sets of primers were tested against the non-redundant (nr) Genbank genetic database constraint to specific phyla: Apicomplexa, Dinoflagellata, Haplosporidians, Thraustochytridae, organisms belonging to *Parvilucifera* genus and *Perkinsus* species. Targets that had six or more mismatches to the primers were ignored. Details of targets presenting less than six mismatches are listed in the table. Number of mismatches between the target and the primer are represented by F to the forward primer and by R to the reverse primer.

Primers	Taxid	Phylum	Nb. of mismatch	Products on target templates	NCBI reference sequence	Product length (bp)
PolsITS2_F/PolsITS2_R	**330153**	***Perkinsus chesapeaki***		**no match sequences**		
	31276	*Perkinsus marinus*	F:5, R:5	*Perkinsus marinus* ATCC 50983 hypothetical protein, mRNA	XM_002787768.1	1082
	259652	*Perkinsus mediterraneus*		no match sequences		
	1074429	*Perkinsus beihaiensis*	F:1, R:4	*Perkinsus beihaiensis* isolate 138 et 31	JX502841.1/JX502840.1	83
	386307	*Perkinsus honshuensis*		no match sequences		
	103982	*Parvilucifera* spp.		no match sequences		
	5794	Apicomplexa	F:4, R:4	*Plasmodium knowlesi* strain H SICA antigen (fragment) partial mRNA	XM_002259474.1	2294
			F:5, R:4	*Plasmodium cynomolgi* genome assembly, chromosome: 7	LT841385.1	1027
			F:5, R:4	*Gregarina niphandrodes* peptidase partial mRNA	XM_011133033.1	2460
	2864	Dinoflagellates		no match sequences		
	31291	Haplosporidians		no match sequences		
	33674	Thraustochytridae		no match sequences		
PchesITS2_F/PchesITS2_R	**32597**	***Perkinsus olseni***		**no match sequences**		
	31276	*Perkinsus marinus*		no match sequences		
	259652	*Perkinsus mediterraneus*		no match sequences		
	1074429	*Perkinsus beihaiensis*		no match sequences		
	386307	*Perkinsus honshuensis*		no match sequences		
	103982	*Parvilucifera* spp.		no match sequences		
	5794	Apicomplexa	F:5, R:5	*Neospora caninum* Liverpool, chromosome chrXI, complete genome	LN714486.1	3944
	2864	Dinoflagellates		no match sequences		
	31291	Haplosporidians		no match sequences		
	33674	Thraustochytridae		no match sequences		

Table S4. Ten best hits from the *in silico* specificity test of (A) *P. olseni* and (B) *P. chesapeaki* probes using Blastn against nr NCBI database (accessed March 2019) excluding the corresponding parasitic sequences respectively. Best hits were dominated by *Perkinsus* sp. sequences, marked by * down to both tables due to poor affiliation in the reference database. These sequences were fully checked using Blastn and respectively affiliated to *P. olseni* and *P. chesapeaki* species; thereafter they were discarded to the 10 Best hits.

A.

10 Best hits for *P. olseni* probe sequence						
Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
PREDICTED: *Quercus suber* beta-mannosidase A-like (LOC112014742), mRNA	36.2	36.2	90%	8.3	100%	XM_024047133.1
Rhodococcus sp. DMU1 chromosome, complete genome	34.2	34.2	85%	33	100%	CP050952.1
Monaibacterium sp. ALG8 chromosome, complete genome	34.2	34.2	85%	33	100%	CP049811.1
Halogeometricum borinquense strain wsp4 chromosome, complete genome	34.2	34.2	85%	33	100%	CP048739.1
Salmo trutta genome assembly, chromosome: 12	34.2	34.2	85%	33	100%	LR584441.1
Salmo trutta genome assembly, chromosome: 29	34.2	34.2	85%	33	100%	LR584418.1
Bos mutus isolate yakQH1 chromosome 22	34.2	34.2	85%	33	100%	CP027090.1
Ovis canadensis canadensis isolate 43U chromosome 19 sequence	34.2	34.2	85%	33	100%	CP011904.1
Rhodococcus aetherivorans strain IcdP1, complete genome	34.2	34.2	85%	33	100%	CP011341.1
PREDICTED: *Callorhinchus milii* cytosolic phospholipase A2 gamma-like (LOC103190768), transcript variant X1, mRNA	34.2	34.2	85%	33	100%	XM_007911657.1
* *Perkinsus* sp. : KM983404, LC524156, AF522321, U07699, U07698, KM983403, KM983402, KM983401	99.52% to 100% : *P. olseni*					

B.

10 Best hits for *P. chesapeaki* probe sequence						
Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Arabis alpina genome assembly, chromosome: 5	40.1	40.1	90%	0.53	100%	LT669792.1
Arabis alpina genome assembly, chromosome: 4	40.1	152	90%	0.53	100%	LT669791.1
Arabis alpina genome assembly, chromosome: 2	40.1	199	90%	0.53	100%	LT669789.1
Microbacterium sp. SGAir0570 chromosome, complete genome	36.2	36.2	81%	8.3	100%	CP027929.1
Cellulomonas shaoxiangyii strain Z28 chromosome, complete genome	36.2	36.2	81%	8.3	100%	CP039291.1
Halomonas alkaliphila X3, complete sequence	36.2	36.2	81%	8.3	100%	CP024811.1
Paenibacillus lautus strain E7593-69 chromosome, complete genome	36.2	36.2	81%	8.3	100%	CP032412.1
Rubrobacter indicoceani strain SCSIO 08198 chromosome	36.2	36.2	81%	8.3	100%	CP031115.1
Kocuria rhizophila strain NCTC8340 genome assembly, chromosome: 1	36.2	36.2	81%	8.3	100%	LR134409.1
Kocuria sp. BT304 chromosome, complete genome	36.2	36.2	81%	8.3	100%	CP030039.1

* *Perkinsus* sp. : KM983418.1, KM983416.1, KM983415.1, KM983414.1, KM983413.1, 98.93% to 100% : *P. chesapeaki* KM983412.1, KM983411.1, KM983409.1, KM983407.1, KM983406.1, KM983405.1, KM983400.1, JQ669649.1, JQ669647.1, JQ669646.1, AF440466.1, AF440465.1, KM983408.1

Table S5. Standard curve equations, qPCR efficiencies and ANOVA-test (F) associated with p-value (significant '*' 0.05, '**' 0.01, '***' 0.001 and not significant 'ns') for each condition depending on the type of organ and its concentration (ng/µl)) tested on both *P. olseni*-plasmidic and *P. chesapeaki*-plasmidic serial dilutions. Red highlights represent best parameters for *P. olseni*; grey highlights represent best parameters for *P. chesapeaki*.

Species	Type of organ	Organ DNA concentration (ng/µl)	Equation curve	R^2	ANCOVA (F)	p-value	Efficiency ($10^{(-1/slope)}$x100%)
P. olseni	Gill	2	-3.34x + 38.77	0.99	0.00	ns	99.9%
P. olseni	Gill	5	-3.13x + 38.82	0.99	0.01	ns	108.6%
P. olseni	Gill	10	-3.37x + 40.57	0.93	0.10	ns	97.9%
P. olseni	Gill	20	-3.32x + 39.97	0.96	1.85	ns	100.3%
P. olseni	Digestive gland	2	-3.32x + 38.83	0.99	0.01	ns	100.3%
P. olseni	Digestive gland	5	-3.43x + 39.38	1.00	0.52	ns	95.9%
P. olseni	Digestive gland	10	-3.28x + 40.11	0.93	0.09	ns	101.7%
P. olseni	Digestive gland	20	-3.31x + 39.75	0.98	0.82	ns	100.4%
P. olseni	Adductor muscle	2	-3.40x + 39.09	1.00	0.22	ns	97.1%
P. olseni	Adductor muscle	5	-3.36x + 39.00	0.99	0.04	ns	98.6%
P. olseni	Adductor muscle	10	-3.13x + 40.27	0.84	2.88	ns	108.7%
P. olseni	Adductor muscle	20	-3.00x + 38.97	0.97	5.54	*	115.4%
P. olseni	Foot	2	-3.57x + 40.22	0.99	4.47	*	90.6%
P. olseni	Foot	5	-3.51x + 40.36	0.97	1.61	ns	92.8%
P. olseni	Foot	10	-2.54x + 39.70	0.47	8.43	**	147.3%
P. olseni	Foot	20	-3.26x + 41.27	0.91	17.59	***	102.7%
P. olseni	Mantle	2	-3.53x + 40.07	0.99	2.43	ns	92.0%
P. olseni	Mantle	5	-3.57x + 41.46	0.94	2.28	ns	90.6%
P. olseni	Mantle	10	-1.83x + 37.25	0.24	16.04	***	252.2%
P. olseni	Mantle	20	-3.05x + 42.76	0.90	2.84	ns	112.9%
P. olseni	Remaining tissue	2	-3.19x + 38.46	0.99	1.06	ns	105.6%
P. olseni	Remaining tissue	5	-2.99x + 42.07	0.55	6.11	*	115.9%
P. olseni	Remaining tissue	10	-2.89x + 41.55	0.68	2.16	ns	121.7%
P. olseni	Remaining tissue	20	-3.07x + 44.28	0.93	8.71	**	111.9%
P. chesapeaki	Gill	2	-3.38x + 39.71	0.99	0.00	ns	97.4%
P. chesapeaki	Gill	5	-3.44x + 40.18	0.98	0.15	ns	95.4%
P. chesapeaki	Gill	10	-3.50x + 41.89	0.94	0.65	ns	93.1%
P. chesapeaki	Gill	20	-3.36x + 42.12	0.95	0.01	ns	98.3%
P. chesapeaki	Digestive gland	2	-3.47x + 40.18	0.99	0.42	ns	94.0%
P. chesapeaki	Digestive gland	5	-3.53x + 40.52	1.00	1.01	ns	92.2%
P. chesapeaki	Digestive gland	10	-3.51x + 41.97	0.95	0.92	ns	92.5%
P. chesapeaki	Digestive gland	20	-3.39x + 41.97	0.96	0.01	ns	97.2%
P. chesapeaki	Adductor muscle	2	-3.43x + 39.94	0.99	0.12	ns	95.6%
P. chesapeaki	Adductor muscle	5	-3.41x + 40.03	0.99	0.06	ns	96.3%
P. chesapeaki	Adductor muscle	10	-3.09x + 40.84	0.85	5.47	*	110.5%
P. chesapeaki	Adductor muscle	20	-3.15 + 40.69	0.97	2.34	ns	107.8%
P. chesapeaki	Foot	2	-3.64x + 41.08	0.99	2.22	ns	88.3%
P. chesapeaki	Foot	5	-3.61x + 41.83	0.95	1.98	ns	89.3%
P. chesapeaki	Foot	10	-2.32x + 39.39	0.40	24.56	***	169.6%
P. chesapeaki	Foot	20	-3.37x + 42.98	0.98	16.08	***	98.2%
P. chesapeaki	Mantle	2	-3.53x + 40.80	0.99	4.60	ns	92.1%
P. chesapeaki	Mantle	5	-3.51x + 42.26	0.93	1.50	ns	92.6%
P. chesapeaki	Mantle	10	-2.15x + 38.31	0.45	11.29	**	192.3%
P. chesapeaki	Mantle	20	-2.75x + 42.59	0.89	12.46	***	130.8%
P. chesapeaki	Remaining tissue	2	-3.43x + 40.56	0.98	0.12	ns	95.7%
P. chesapeaki	Remaining tissue	5	-2.45x + 40.49	0.38	39.09	***	156.4%
P. chesapeaki	Remaining tissue	10	-2.80x + 42.53	0.64	5.65	*	127.4%
P. chesapeaki	Remaining tissue	20	-3.26x + 46.87	0.91	13.55	***	102.5%

References.

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

Brown. G. D.. Hudson. K. L. and Reece. K. S. (2004). Multiple polymorphic sites at the ITS and ATAN loci in cultured isolates of *Perkinsus marinus*. *Journal of Eukaryotic Microbiology* **51**. 312–320. doi: 10.1111/j.1550-7408.2004.tb00572.x.

Burreson. E. M.. Reece. K. S. and Dungan. C. F. (2005). Molecular. morphological. and experimental evidence support the synonymy of *Perkinsus chesapeaki* and *Perkinsus andrewsi*. *Journal of Eukaryotic Microbiology* **52**. 258–270. doi: 10.1111/j.1550-7408.2005.05-00035.x.

Casas. S. M.. Grau. A.. Reece. K. S.. Apakupakul. K.. Azevedo. C. and Villalba. A. (2004). *Perkinsus mediterraneus* n. sp.. a protistan parasite of the European flat oyster *Ostrea edulis* from the Balearic Islands. Mediterranean Sea. *Diseases of Aquatic Organisms* **58**. 231–244. doi: 10.3354/dao058231.

Casas. S. M.. Reece. K. S.. Li. Y.. Moss. J. A.. Villalba. A. and La Peyre. J. F. (2008). Continuous culture of *Perkinsus mediterraneus*. a parasite of the European flat oyster *Ostrea edulis*. and characterization of its morphology. propagation. and extracellular proteins *in vitro*. *Journal of Eukaryotic Microbiology* **55**. 34–43. doi: 10.1111/j.1550-7408.2008.00301.x.

Choi. K.-S.. Wilson. E. A.. Lewis. D. H.. Powell. E. N. and Ray. S. M. (1989). The energetic cost of *Perkinsus marinus* parasitism in oysters: quantification of the thioglycollate method. 125–131.

da Silva. P. M.. Vianna. R. T.. Guertler. C.. Ferreira. L. P.. Santana. L. N.. Fernández-Boo. S.. Ramilo. A.. Cao. A. and Villalba. A. (2013). First report of the protozoan parasite *Perkinsus marinus* in South America. infecting mangrove oysters *Crassostrea rhizophorae* from the Paraíba River (NE. Brazil). *Journal of Invertebrate Pathology* **113**. 96–103. doi: 10.1016/j.jip.2013.02.002.

da Silva. P. M.. Scardua. M. P.. Vianna. R. T.. Mendonça. R. C.. Vieira. C. B.. Dungan. C. F.. Scott. G. P. and Reece. K. S. (2014). Two *Perkinsus* spp. infect *Crassostrea gasar* oysters from cultured and wild populations of the Rio São Francisco estuary. Sergipe. northeastern Brazil. *Journal of Invertebrate Pathology* **119**. 62–71. doi: 10.1016/j.jip.2014.04.005.

Dungan. C. F. and Reece. K. S. (2006). *In Vitro* propagation of two *Perkinsus* spp. parasites from Japanese Manila clams *Venerupis philippinarum* and description of *Perkinsus honshuensis* n. sp. *Journal of Eukaryotic Microbiology* **53**. 316–326. doi: 10.1111/j.1550-7408.2006.00120.x.

Elandaloussi. L. M.. Carrasco. N.. Roque. A.. Andree. K. and Dolores Furones. M. (2009). First record of *Perkinsus olseni*. a protozoan parasite infecting the commercial clam *Ruditapes decussatus* in Spanish Mediterranean waters. *Journal of Invertebrate Pathology* **100**. 50–53. doi: 10.1016/j.jip.2008.09.004.

Itoh. N.. Meyer. G.. Tabata. A.. Lowe. G.. Abbott. C. and Johnson. S. (2013). Rediscovery of the Yesso scallop pathogen *Perkinsus qugwadi* in Canada. and development of PCR tests. *Diseases of Aquatic Organisms* **104**. 83–91. doi: 10.3354/dao02578.

Moss. J. A.. Xiao. J.. Dungan. C. F. and Reece. K. S. (2008). Description of *Perkinsus beihaiensis* n. sp.. a new *Perkinsus* sp. parasite in oysters of southern China. *Journal of Eukaryotic Microbiology* **55**. 117–130. doi: 10.1111/j.1550-7408.2008.00314.x.

Queiroga. F. R.. Vianna. R. T.. Vieira. C. B.. Farias. N. D. and Da Silva. P. M. (2015). Parasites infecting the cultured oyster *Crassostrea gasar* (Adanson. 1757) in northeast Brazil. *Parasitology* **142**. 756–766. doi: 10.1017/S0031182014001863.

Ramilo. A.. Carrasco. N.. Reece. K. S.. Valencia. J. M.. Grau. A.. Aceituno. P.. Rojas. M.. Gairin. I.. Furones. M. D.. Abollo. E. and Villalba. A. (2015). Update of information on perkinsosis in NW Mediterranean coast: Identification of *Perkinsus* spp. (Protista) in new locations and hosts. *Journal of Invertebrate Pathology* **125**. 37–41. doi: 10.1016/j.jip.2014.12.008.

Ramilo. A.. Pintado. J.. Villalba. A. and Abollo. E. (2016). *Perkinsus olseni* and *P. chesapeaki* detected in a survey of perkinsosis of various clam species in Galicia (NW Spain) using PCR–DGGE as a screening tool. *Journal of Invertebrate Pathology* **133**. 50–58. doi: 10.1016/j.jip.2015.11.012.

Reece. K.. Dungan. C. and Burreson. E. (2008). Molecular epizootiology of *Perkinsus marinus* and *P. chesapeaki* infections among wild oysters and clams in Chesapeake bay. USA. *Diseases of Aquatic Organisms* **82**. 237–248. doi: 10.3354/dao01997.

Reece. K. S.. Scott. G. P.. Dang. C. and Dungan. C. F. (2017). A novel monoclonal *Perkinsus chesapeaki in vitro* isolate from an Australian cockle. *Anadara trapezia*. *Journal of Invertebrate Pathology* **148**. 86–93. doi: 10.1016/j.jip.2017.05.007.

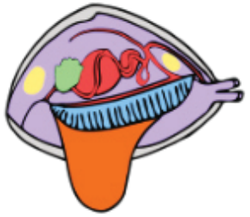
Sabry. R. C.. Gesteira. T. C. V.. Magalhães. A. R. M.. Barracco. M. A.. Guertler. C.. Ferreira. L. P.. Vianna. R. T. and da Silva. P. M. (2013). Parasitological survey of mangrove oyster. *Crassostrea rhizophorae*. in the Pacoti river estuary. Ceará state. Brazil. *Journal of Invertebrate Pathology* **112**. 24–32. doi: 10.1016/j.jip.2012.10.004.

Shamal. P.. Zacharia. P. U.. Binesh. C. P.. Pranav. P.. Suja. G.. Asokan. P. K.. Pradeep. M. A.. Rithesh. R.. Vijayan. K. K. and Sanil. N. K. (2018). *Perkinsus olseni* in the short neck yellow clam. *Paphia malabarica* (Chemnitz. 1782) from the southwest coast of India. *Journal of Invertebrate Pathology* **159**. 113–120. doi: 10.1016/j.jip.2018.10.001.

Valencia. J. M.. Bassitta. M.. Picornell. A.. Ramon. C. and Castro. J. A. (2014). New data on *Perkinsus mediterraneus* in the Balearic archipelago: locations and affected species. *Diseases of Aquatic Organisms* **112**. 69–82. doi : 10.3354/dao02795.

Ye. J.. Coulouris. G.. Zaretskaya. I.. Cutcutache. I.. Rozen. S. and Madden. T. L. (2012). Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics* **13**. 1–11. doi: 10.1186/1471-2105-13-134.

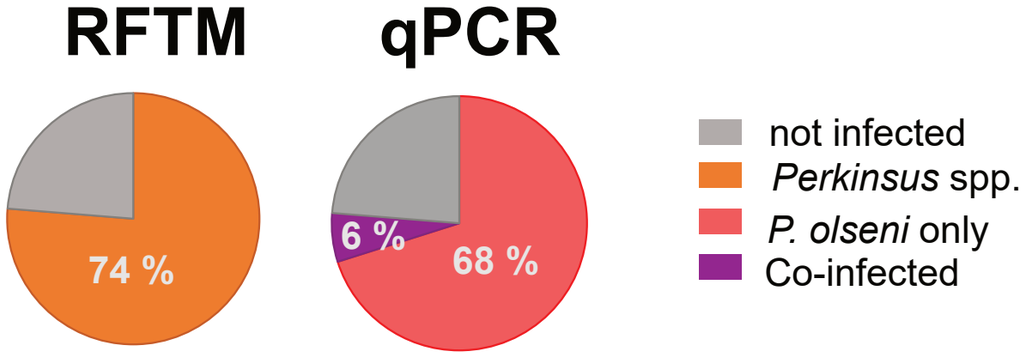
1 Duplex qPCR assay development



- Gill
- Digestive gland
- Mantle
- Foot
- Adductor muscles
- Remaining tissue

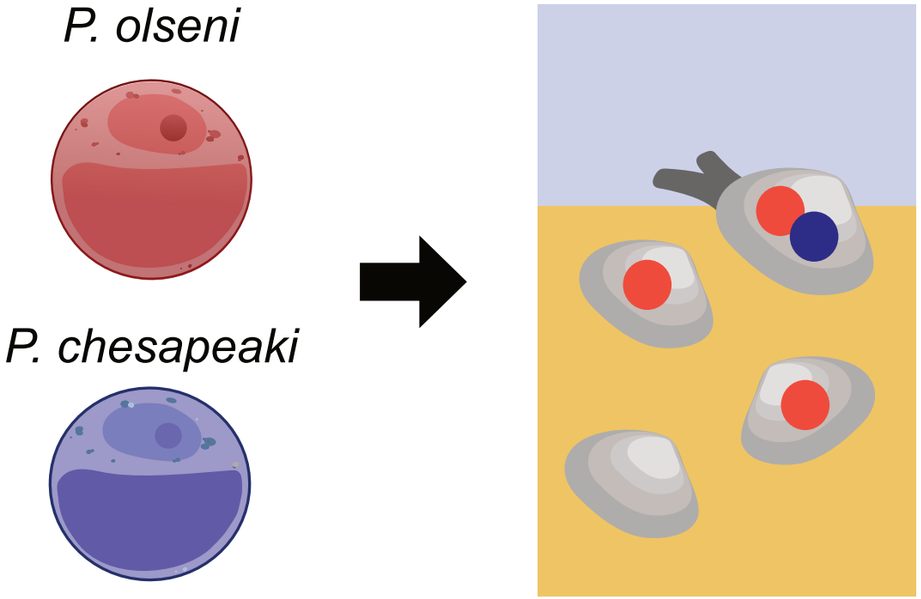
⏩ Presence of PCR inhibitors depending on organ-type

2 Comparison RFTM and qPCR methods



⏩ RFTM and qPCR are complementary but only qPCR can quantify abundance at species level

3 *In situ* co-detection and quantification



⏩ Single-infection was only detected *for P. olseni* parasite.

⏩ The infection intensities of co-infected organisms are dominated by *P. olseni* parasite.