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Sarah Itoiz

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Spécialité : « *Biologie marine* »

Par

Sarah ITOIZ

Ecologie fonctionnelle des Perkinsozoa (Alveolata) en milieu marin : de la diversité globale à l'étude d'un cas de co-infection entre *Perkinsus olseni* et *Perkinsus chesapeaki*.

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« Alors, quelque chose de tookien s'éveilla en lui, il souhaita aller voir les grandes montagnes, entendre les pins et les cascades, explorer les cavernes et porter une épée au lieu d'une canne »

J.R.R Tolkien

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Liste des abréviations

ADN : Acide désoxyribonucléique

ARN : Acide ribonucléique

ASV : « Amplicon sequence variant »

DOM : « Dissolved organic matter » = Matière organique dissoute

EID : « Emerging Infectious Disease » = Maladie émergente infectieuse

EMM : Evénements de Mortalités Massives

eToL: « Eukaryotic tree of life »

HAB: « Harmful algal bloom »= Efflorescence algal toxique

ITS: « Internal Transcribed Spacer »

MALV: « Marine Alveolate »

MAST: « Marine Stramenopile »

MHC: « Major histocompatibility complex »

NGS : « Next Generation Sequencing »

NTS: « Nontranscribed Spacer »

OTU : « Operational taxonomic unit » = Unité taxonomique opérationnelle

O.I.E : Organisation mondiale de la santé animale

POM : « Particulate organic matter » = Matière organique particulaire

SIDA : Syndrome d'immunodéficience acquise

Th2 : cytokines « T-helper 2 »

INTRODUCTION

Les relations symbiotiques, ou « interactions durables » (Combes, 1995), désignent des relations inter-organismes étroites, inscrites dans la durée, avec ou sans principe de réciprocité (De Bary, 1879; Combes, 1995; Combes *et al.*, 2018). Ces interactions peuvent ainsi être antagonistes, neutres ou bénéfiques pour l'un et/ou l'autre des partenaires. Elles se définissent sur un continuum allant de la relation parasitaire, où l'interaction est bénéfique pour l'un des partenaires et néfaste pour l'autre, à la relation mutualiste, où chaque partenaire tire bénéfice de l'interaction (Figure 1).

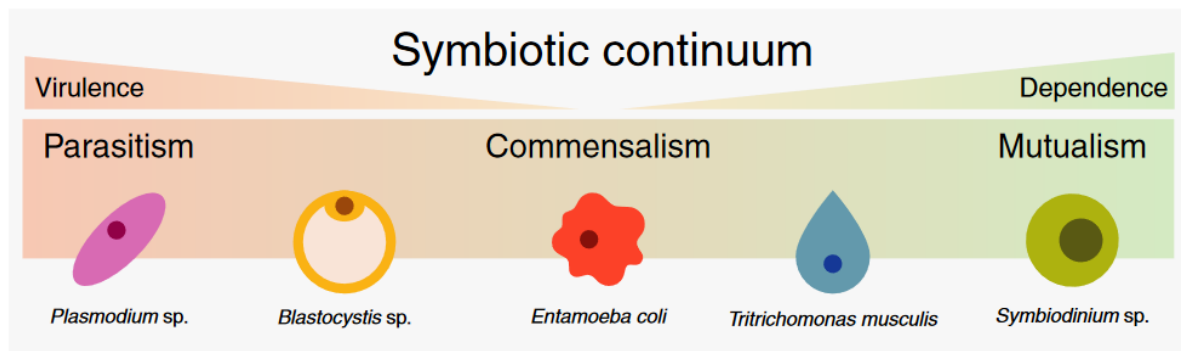


Figure 1. Spectre symbiotique issu de del Campo *et al.* (2020) allant du parasitisme au mutualisme en passant par le commensalisme. Le parasitisme et le mutualisme sont souvent considérés comme deux opposés sur ce continuum mais cela n'empêche pas certains symbiotes de basculer d'une interaction à une autre.

Les limites de ces interactions ne sont pas fixes dans le temps et dans l'espace mais sont dynamiques, ainsi il est possible d'observer chez certains organismes un passage du statut de parasite à celui de mutualiste et vice versa (Douglas, 2008). Ces transitions peuvent se produire en réponse à des changements de leur environnement comme cela a déjà été observé chez les symbiotes de coraux *Ostreobium* spp. (Verbruggen and Tribollet, 2011) ou encore *Symbiodinium* spp. (Baker *et al.*, 2018). En effet, l'augmentation des températures et l'eutrophisation des habitats (= accumulation de nutriments comme l'azote ou le phosphore) modifient l'équilibre dans la relation mutualiste hôte-symbiote et participent largement au phénomène de blanchiment chez les coraux (Weis, 2008; Cunning and Baker, 2013; Vega Thurber *et al.*, 2014). Dans ces conditions, *Symbiodinium* tend à augmenter la séquestration des ressources pour sa propre croissance, passant ainsi à l'état de parasite dans l'hôte qui l'abrite

(Baker *et al.*, 2018). Ces relations symbiotiques (parasitisme, commensalisme et mutualisme) sont de véritables forces évolutives et structurales au sein des écosystèmes marins et terrestres. Ces liens se tissent entre toutes les formes du vivant (Tableau 1).

Tableau 2. Exemples d'interaction entre différents groupes d'organismes montrant leur diversité et leur interconnexion. Cette liste n'est pas exhaustive.

Organismes impliqués		Types d'interaction	Exemples	Références
1	2			
Métazoaire	Métazoaire	Parasitisme	Infestation de l'Homme par des nématodes (e.g <i>Necator americanus</i> et <i>Ankylostoma duodenale</i>)	Loukas <i>et al.</i> (2016)
Plante	Bactérie	Mutualisme	Symbiose rhizobienne impliquant des bactéries diazotrophes (e.g <i>Frankia</i> sp. et des cyanobactéries)	Santi <i>et al.</i> (2013)
Plante	Fungi ¹	Mutualisme	Symbiose mycorhizienne (= 90 % des plantes terrestres)	Smith <i>et al.</i> (2009)
Métazoaire	Protiste	Parasitisme/ Mutualisme	Présence de certains protistes dans les intestins humain (e.g <i>Blastocystis</i>)	Parfrey <i>et al.</i> (2014)
Protiste ²	Protiste	Parasitisme	Infection lors de blooms phytoplanctoniques (e.g <i>Amoebophrya</i> infectant des dinoflagellés)	Chambouvet <i>et al.</i> , (2008)
Protiste	Fungi	Parasitisme	Infection de diatomées par des Opisthosporidia (e.g NCLC1)	Chambouvet <i>et al.</i> , (2019)
Bactérie	Métazoaire	Mutualisme	les modioles (Mytilidae: Bathymodiolinae) et leurs bactéries chimio-synthétiques	Duperron <i>et al.</i> , (2009)
Bactérie	Protiste	Mutualisme	Ciliés (Euplotes) et leurs symbiontes bactériens obligatoires du genre <i>Polynucleobacter</i>	Vannini <i>et al.</i> , (2012)

Ces exemples ne sont qu'un bref aperçu des interconnexions existant au sein des réseaux trophiques. Beaucoup de ces relations symbiotiques impliquent des micro-organismes, qui malgré leur petite taille, occupent une partie importante de la biomasse globale (93 Gt de

¹ **Fungi** : groupe monophylétique relatif aux Holomycota (Opisthokonta) et frère des Holozoa. La démarcation entre Protiste et Fungi pourrait reposer sur quelques traits physiologiques comme l'apparition de la paroi chitineuse et la perte de la phagotrophie (Ruggiero *et al.*, 2015). Il n'y a pas de consensus sur la définition et la composition du règne des Fungi notamment à cause du manque de résolutions sur les branches les plus basales (e.g. Microsporidia, Aphelida) (Richards *et al.*, 2017).

² **Protiste** : groupe paraphylétique composé de sept phylum principaux (Ruggiero *et al.*, 2015) défini par Adl *et al.*, (2005, 2007) de la façon suivante : « organismes eucaryotes dotés d'une organisation unicellulaire, coloniale, filamenteuse ou parenchymateuse dépourvus de différenciation tissulaire, sauf pour la reproduction ». Dans cette définition, Adl *et al.* (2005) inclut les Fungi comme membre à part entière. Cependant, ici, nous séparons les deux règnes car les Fungi présentent une différenciation graduelle avec les Protistes comme l'a déterminé Richards *et al.* en 2017.

carbone) comparée à celle des animaux (2 Gt de carbone) (Bar-On *et al.*, 2018). Leur place est ainsi primordiale dans la structuration des réseaux trophiques (Worden *et al.*, 2015).

1. Le parasitisme : une constante essentielle des écosystèmes

Chaque organisme sur Terre héberge au moins une espèce de parasite (Poulin and Morand, 2000). Le parasitisme est un mode trophique ubiquitaire pouvant représenter jusqu'à 78% des interactions trophiques au sein d'un écosystème (Lafferty *et al.*, 2006). Plusieurs études ont estimé que les parasites représentent entre 30 à 71% de la diversité décrite de la biosphère (~71%-Price, 1980; ~50%-De Meeûs *et al.*, 1998 ; ~30%-De Meeûs and Renaud, 2002; ~40%-Dobson *et al.*, 2008). Selon les archives paléontologiques, ce mode de vie montre ses premières traces à la fin du Cambrien (-497 à -485 MA) (Sorci and Garnier, 2019) et serait apparu de façon indépendante dans les différentes ramifications du vivant expliquant ainsi leur diversité foisonnante (De Meeûs and Renaud, 2002; Weinstein and Kuris, 2016). Les parasites arborent une grande pluralité dans leur phylogénie, leur forme, leur cycle de vie, leur mode de transmission ou encore leur spectre d'hôte.

1.1. Caractérisation des parasites

1.1.1 Macro- et micro-parasites

Les parasites sont séparés traditionnellement suivant un critère de taille donnant naissance à deux grands groupes : les macroparasites, incluant les helminthes et les arthropodes, puis les micro-parasites, incluant les fungi, les protistes, les bactéries et les virus (Figure 2). Les macroparasites sont des parasites métazoaires ayant une durée de vie relativement longue qui produisent des stades infectieux libres permettant d'infecter de nouveaux hôtes. Bien souvent, la relation entre ces organismes et la réponse immunitaire de l'hôte se traduit par une course de fond amenant les macroparasites à faire appel à des stratégies de "déguisement", de protection et de modulation du système immunitaire pour leur survie (Viney and Cable, 2011). Ces stratégies résultent généralement d'une longue durée de vie de ces parasites, en dépit des réponses immunitaires de l'hôte se développant plus lentement et étant partiellement efficaces (Jackson *et al.*, 2004; Viney and Cable, 2011). A l'inverse, les micro-parasites sont des organismes unicellulaires ayant un temps de génération relativement court avec une forte

multiplication dans l'hôte et une tendance à produire une réponse immunitaire forte chez les hôtes infectés (Anderson and May, 1979).



Figure 2. Dichotomie des parasites en fonction de leur taille : les macroparasites (helminthes et arthropodes) et les microparasites (fungi, protistes, bactéries, virus) d'après Anderson et May (1979).

1.1.2 Cycle de vie et spectre d'hôtes

- **La complexité des cycles de vie parasitaires**

Ces deux types de parasite peuvent réaliser leur cycle de vie en passant d'un hôte à l'autre, soit directement en l'absence d'hôte intermédiaire (cycle monoxène³) (e.g les espèces du genre *Cryptosporidium* n'ont besoin que d'un seul hôte, un vertébré (e.g Homme) (Fayer, 2004)), soit indirectement par l'intermédiaire d'une ou plusieurs espèces d'hôte intermédiaire essentielles pour son développement (cycle hétéroxène⁴) (e.g *Trypanosoma brucei* a besoin de deux hôtes pour se développer, la mouche tsé-tsé (*Glossina palpalis*) et un vertébré (Homme, bovidés, antilopes)(Wamwiri and Changasi, 2016)). L'hôte intermédiaire ne doit pas être confondu avec la notion d'hôte parénétique, en effet, le premier est essentiel au cycle de vie du parasite et y abrite un stade de son développement tandis que l'autre n'est pas obligatoire et ne remplit qu'une fonction de transport (Behrmann-Godel, 2015). La transmission directe peut se faire par contact physique entre hôtes comme les infections par voie sexuelle observées chez les mammifères (Mastro and De Vincenzi, 1996) ou encore les insectes (Knell and Webberley, 2004) ; par inhalation de l'agent infectieux comme le SARS-CoV-2 (Vuorinen *et al.*, 2020) ;

³ **Monoxène** (syn. Holoxène) : cycle de vie parasitaire où n'intervient qu'un seul hôte dans sa réalisation

⁴ **Hétéroxène** : cycle de vie parasitaire où intervient deux hôte ou plus dans sa réalisation

par ingestion de l'agent étiologique comme celui responsable de la giardiose (*Giardia* spp.) qui se transmet via la consommation d'éléments contaminés e.g. l'eau et la nourriture (Hunter and Thompson, 2005); ou encore par filtration chez les mollusques comme c'est le cas pour les parasites du genre *Perkinsus* (Villalba *et al.*, 2004). La transmission indirecte est plus complexe. Elle peut impliquer la piqûre ou la morsure par des vecteurs (mouches, moustiques, tiques et autres). Le plus connu des genres possédant ce type de transmission est le micro-parasite *Plasmodium*, agent étiologique du paludisme (syn. Malaria), responsable de 500 millions de décès en 2015 à travers le monde. Le moustique (femelle), *Anopheles* sp., qui se trouve être l'hôte définitif transmet le parasite à son hôte intermédiaire l'Homme (Howick *et al.*, 2019). La transmission indirecte peut aussi se réaliser par la pénétration de stades libres qui sont produits par des hôtes intermédiaires comme cela est le cas pour la bilharziose causée par le trématode *Schistosoma mansoni*. Ce parasite infecte un gastéropode d'eau douce, du genre *Biomphalaria* (hôte intermédiaire), qui abrite le développement des sporocytes et des cercaires. Les cercaires sont relargués dans l'eau et peuvent pénétrer l'épiderme humain causant ainsi une infestation de l'hôte définitif (Vale *et al.*, 2012). Enfin, les parasites peuvent être directement ingérés lorsqu'un hôte intermédiaire infecté est consommé par l'hôte final. C'est le cas dans la toxoplasmose, où *Toxoplasma gondii* se transmet au chat (hôte définitif) par la consommation des rongeurs ou d'oiseaux infectés après ingestion d'éléments contaminés par des oocystes du parasite (Dubey, 1998).

○ La diversité des spectres d'hôtes

La diversité des parasites se retrouve aussi sur les interactions interspécifiques qu'ils entretiennent avec leurs hôtes. Les spectres d'hôtes s'étendent sur un continuum opposant des spectres très larges à d'autres extrêmement restreints. Les parasites présentant des spectres d'hôtes larges sont présents aussi bien chez les macroparasites, par exemple avec le nématode *Trichostrongylus axei* infectant six espèces d'ongulés sauvages (mouflon, cerf, bison, antilope et cerfs) vivant en sympatrie (Archie and Ezenwa, 2011), que chez les micro-parasites, par exemple avec le protiste *Ichthyodinium chabelardi* (Alveolata) infectant plusieurs espèces de poissons et pouvant causer des mortalités chez *Oncorhynchus tshawytscha* et *Salmo salar* (Gleason *et al.*, 2019) ou *Parvilucifera sinerae* infectant plusieurs genres de dinoflagellés dont *Alexandrium*, *Coolia*, *Dinophysis*, *Fragilidium*, *Gambierdiscus*, *Gymnodinium*, *Gyrodinium*, *Heterocapsa*, *Kryptoperidinium*, *Lepidodinium*, *Ostreopsis*, *Pentaparsodinium*, *Protoceratium*, *Scrippsiella* et *Woloszynskia* (Garcés *et al.*, 2013a). Au sein des Alveolata,

certains spectres d'hôtes sont aussi très spécialisés comme chez les parasites du genre *Eimeria*, par exemple *E. falciformis* qui infecte les cellules épithéliales du cæcum et du côlon chez la souris ou encore *E. tenella* qui infecte les intestins du coq, *Gallus gallus* (Heitlinger *et al.*, 2014). La spécificité du spectre d'hôtes est définie comme la capacité d'un parasite à coloniser de nouveaux hôtes les classant ainsi en deux grands groupes : les spécialistes – infectant un hôte spécifique - et les généralistes – infectant un large spectre d'hôtes. Cette notion de spécialisation est une composante essentielle dans la réalisation du cycle de vie parasitaire (Combes, 2001). Le spectre d'hôtes et par conséquent la survie du parasite est grandement modelé par deux mécanismes proposés par Euzet et Combes en 1980 : le filtre de rencontre – rencontre et comportement favorisant la contamination – et le filtre compatibilité – environnement favorable à la survie et tolérance immunitaire (Euzet and Combes, 1980; Combes, 1995) (Figure 3). Des adaptations permettent d'ouvrir ou de fermer ces filtres qui vont être sélectionnées aussi bien par l'hôte que par le parasite. Cette lutte voue les protagonistes à une interminable course aux armements proposée dans l'hypothèse de la reine rouge par Van Valen en 1977. Ces filtres façonnent les relations hôtes-parasites dans le temps et l'espace jouant ainsi une grande part de responsabilité dans le déterminisme du spectre d'hôtes chez les parasites.

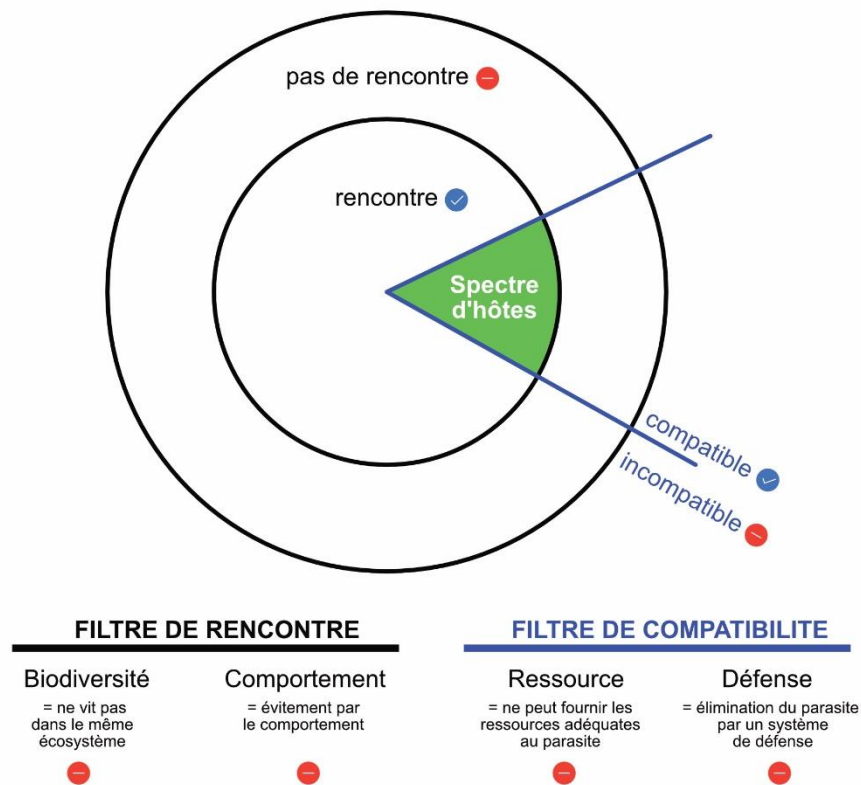


Figure 3. Façonnement du spectre d’hôtes selon Euzet et Combes (1980) adapté de Combes en 2001. L’intersection entre des hôtes compatibles qui rencontrent leur parasite constitue le spectre d’hôtes (section verte). Les filtres de compatibilité et de rencontre sélectionnent la possibilité d’une interaction durable en fonction des éléments cités sous le diagramme.

1.2. L’impact du parasitisme sur les écosystèmes

A l’échelle individuelle, les parasites ont des effets délétères sur la fitness et l’état de santé générale des hôtes (Soares *et al.*, 2014) aussi bien chez les humains (e.g. Kotepui *et al.*, 2020; Weiss *et al.*, 2019), les mammifères marins (e.g. Kemper *et al.*, 2016; Kennedy, 1998) et terrestres (e.g. Feng and Xiao, 2011; Ryan and Cacciò, 2013), les oiseaux (e.g. Remple, 2004), les mollusques (e.g. Choi and Park, 2004; Dove *et al.*, 2004) que les arthropodes (e.g. Grupe and Quandt, 2020). Les charges parasitaires augmentent le risque de maladies infectieuses et de maladies secondaires (Day *et al.*, 2007; Beldomenico *et al.*, 2008).

A l'échelle écosystémique, les parasites s'avèrent être une composante essentielle des milieux marins et terrestres où leur diversité nous renseigne sur la « bonne santé » des écosystèmes (Hudson *et al.*, 2006). Cette diversité parasitaire joue des rôles importants dans :

- (1) **la régulation des populations hôtes**, par exemple au sein des communautés phytoplanctoniques où le contrôle parasitaire (virus, bactéries, protistes, champignons) est important dans le maintien de la succession écologique de différentes espèces (Jacquet *et al.*, 2011). Cette régulation peut être expliquée par l'hypothèse « killing the winner » (Thingstad et Lignell, 1997) selon laquelle les espèces dominantes voient leur biomasse contrôlée tandis que les espèces moins compétitives sont favorisées. C'est ainsi que les parasites modulent **les interactions interspécifiques**, par exemple, *Plasmodium azurophylum* réduit la compétition entre deux espèces d'Anoles (reptile) en infectant préférentiellement l'espèce la plus compétitrice. La diminution de la compétition entre *Anolis gingivinus* et *A. wattsi* permet leur coexistence sur une même aire de répartition (Schall, 1992). Ces régulations favorisent le maintien de la diversité dans les écosystèmes ;

- (2) **les cycles biogéochimiques**, aussi bien par les infections micro-eucaryotes (Worden *et al.*, 2015), virales (Breitbart, 2011) et fongiques (Grami *et al.*, 2011; Sime-Ngando, 2012). La mort d'un hôte parasité entraîne la libération de matière organique particulaire (POM) et dissoute (DOM) participant ainsi activement au cycle du carbone. Par exemple, dans le Lac Pavin, les chytrides parasites de diatomées participent à une augmentation du transfert du carbone dans les échelles trophiques supérieures (zooplancton) et diminuent de 50% l'export de matière organique vers les communautés benthiques (Grami *et al.*, 2011; Frenken *et al.*, 2017)(Figure 4);

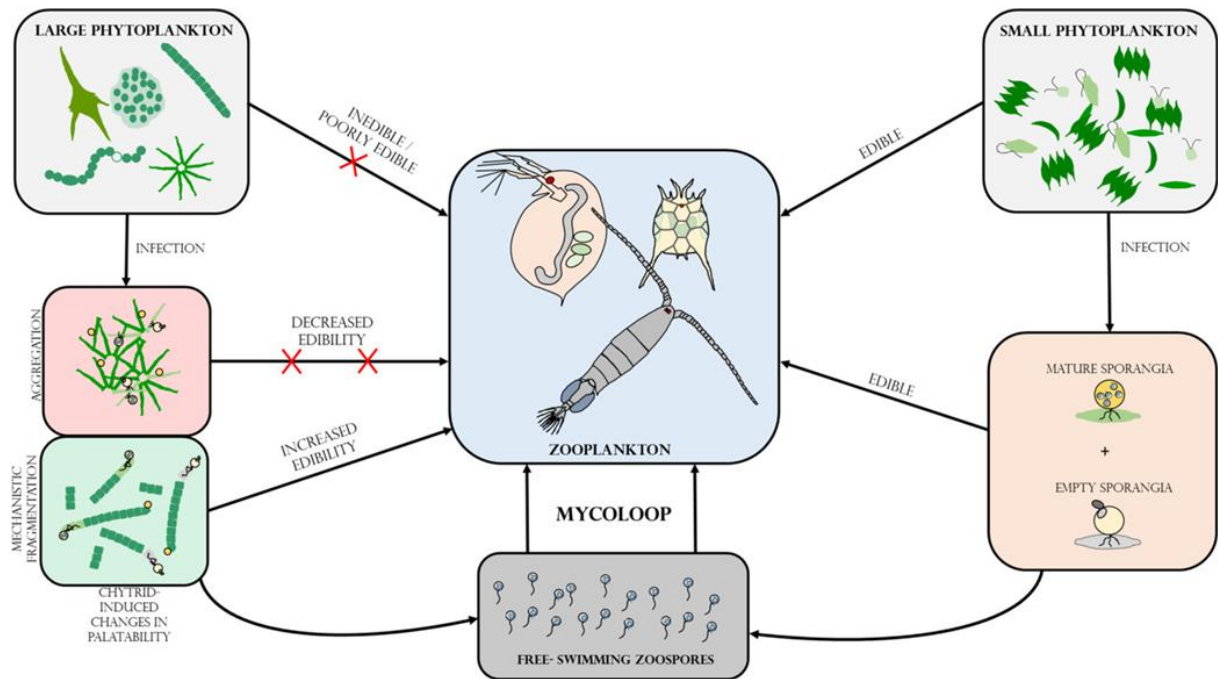


Figure 4. Réseau trophique tiré de Frenken *et al.* (2017) montrant le lien trophique (transfert d'énergie) entre le phytoplancton et le zooplancton par l'intermédiaire de chytrides parasites.

- (3) **la connectivité, la stabilité et la diversité au sein des réseaux trophiques**, en effet de nombreuses études ont suggéré que les parasites augmentent la connectivité et le nombre de liens au sein des réseaux trophiques (Lafferty *et al.*, 2006, 2008; Amundsen *et al.*, 2009; Dunne *et al.*, 2013) ainsi que la richesse spécifique, le nombre de niveaux trophiques et la longueur de ses chaînes (Huxham *et al.*, 1995; Thompson *et al.*, 2005; Amundsen *et al.*, 2009)(Figure 5). La modulation de ces paramètres par les parasites participe à la stabilisation des communautés (Hudson *et al.*, 2006). Cependant, l'hypothèse d'une augmentation de la stabilité des réseaux trophiques dans le cas de l'ajout de parasites reste débattue car l'augmentation de la taille et de la complexité de ces réseaux pencheraient en faveur d'une perte de stabilité (May, 1972).

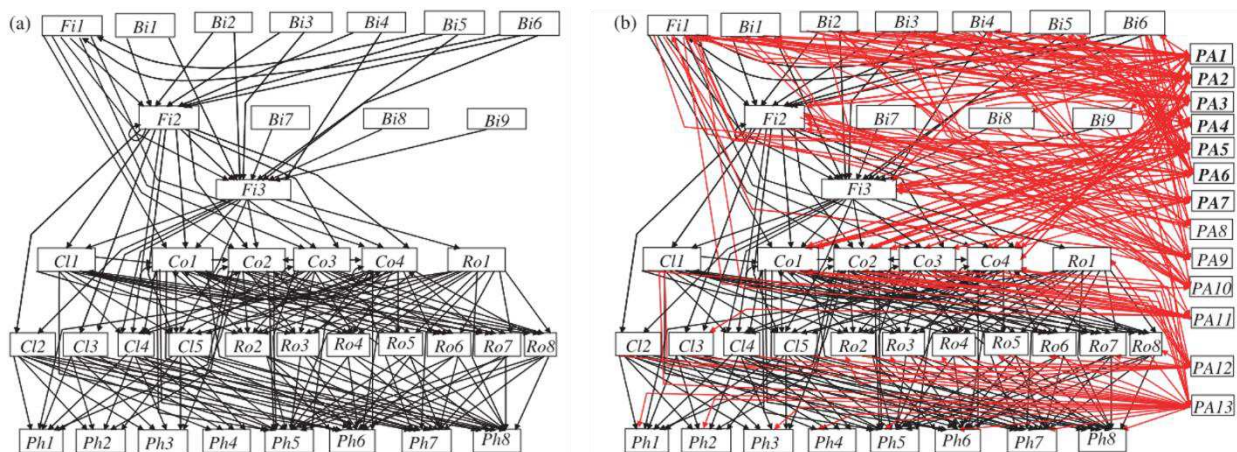


Figure 5. Représentation schématique d'un réseau trophique pélagique subarctique du lac de Takvatn (Norvège) tiré de Amundsen *et al.* (2009). (a) Sans et (b) avec les liens interactions parasitaires (en rouge) ; Bi, oiseaux ; Cl cladocères ; Co, Copépodes ; Fi, poissons ; PA, parasites ; Ph, phytoplancton ; et Ro, rotifères.

Aujourd'hui, les parasites ont mauvaise presse même si leur rôle essentiel dans les écosystèmes n'est plus à prouver. Malgré d'importants services écosystémiques rendus, en 2070, 5 à 10% des parasites pourraient s'éteindre dû à la perte d'habitat ; une conséquence directe du changement climatique (Carlson *et al.*, 2017). En effet, les relations hôte-parasite se retrouvent menacées par les activités anthropiques (pollution, urbanisation, introduction d'espèce) et par l'effet du changement climatique (Harvell *et al.*, 1999). Ces facteurs de stress contribuent à l'expansion et/ou la disparition de certains parasites pouvant avoir des conséquences néfastes sur la santé Humaine et animale (épidémie, zoonose) ainsi que sur la biodiversité (extinction d'espèce, perte de fonction écosystémique). Alors que serait un écosystème sans ses parasites ? En 2007, Wood *et al.* ont formulé plusieurs conséquences hypothétiques de telles disparitions comme : la baisse de régulation de certaines populations hôtes, le remodelage des communautés, la perte de stabilité dans les réseaux trophiques, des modifications dans les flux d'énergie et des phénomènes d'invasions dans certains écosystèmes. De plus, le déclin d'un parasite peut laisser une niche écologique vacante pouvant représenter une opportunité d'installation pour de nouveaux parasites (Lloyd-Smith, 2013) dont les conséquences seraient encore inconnues.

1.3. Les micro-parasites : un maillon clé à la diversité encore mal caractérisée ?

L'avènement de la biologie moléculaire et du séquençage haut-débit a révolutionné la conception de la diversité microbienne avec la (re-) découverte et la (re-)caractérisation de nombreuses espèces de micro-eucaryotes dont de nombreux parasites (Moon-van der Staay *et al.*, 2001; López-García *et al.*, 2003; Massana *et al.*, 2004; Chambouvet *et al.*, 2008; Caron *et al.*, 2012; Chambouvet *et al.*, 2014; De Vargas *et al.*, 2015)(Figure 6).

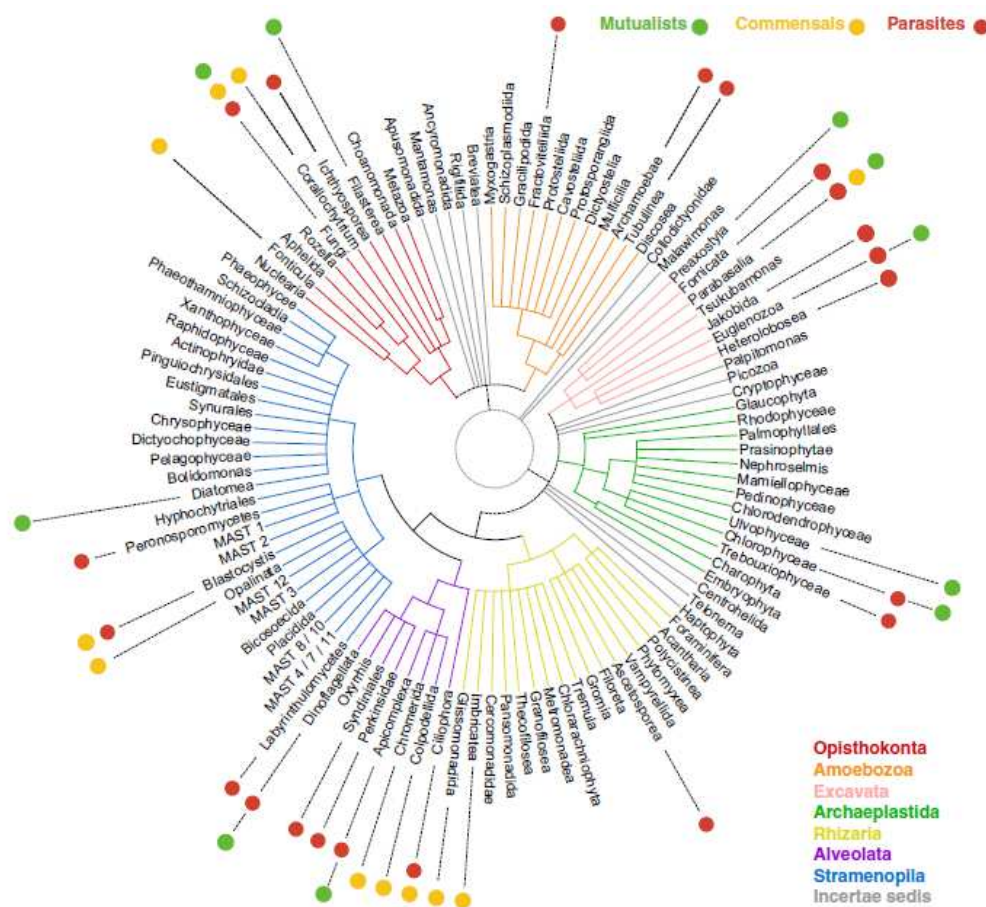


Figure 6. Arbre des eucaryotes montrant tous les groupes contenant des symbiotes micro-eucaryotes connus (mutualistes, parasites et commensaux) tiré de del Campo *et al.* (2020). Les types d'interactions sont représentés par des cercles rouges pour le parasitisme, jaune pour le commensalisme et vert pour le mutualisme. Les branches de cet arbre sont colorées en fonction des grandes lignées de protistes déjà décrites.

Pendant longtemps, la diversité des micro-parasites eucaryotes est restée sous-représentée car difficile d'accès et ne présentant pas un intérêt prioritaire dans le domaine de la santé humaine. Cependant, il paraît clair que ces parasites présentent des rôles essentiels à l'échelle des communautés et des écosystèmes comme énumérés précédemment. Certains groupes de parasites, comme les MALV (Guillou *et al.*, 2008; De Vargas *et al.*, 2015), bénéficient de l'essor des études environnementales et voient leur diversité de mieux en mieux référencée dans les banques de données génétiques. Cependant, beaucoup d'autres micro-parasites, comme les Perkinsozoa, sont encore sous-représentés car généralement retrouvés dans des abondances relativement faibles par rapport aux communautés micro-eucaryotes globales. Bass et del Campo (2020) avancent deux explications concernant ces clades sous-estimés: le biais de PCR et l'association étroite de l'hôte avec son parasite qui le rend difficilement détectable voire quasiment absent des échantillons environnementaux (Bass *et al.*, 2015; Bass and del Campo, 2020). De plus, cette diversité même lorsqu'elle est phylogénétiquement affiliée à des micro-parasites connus, ou retrouvée au sein d'un autre organisme, ne nous permet pas toujours de trancher en faveur d'un mode de vie parasitaire car cela ne peut être confirmé sans preuves de l'infection (postulat de Koch) (Bass and del Campo, 2020). Néanmoins, au cours de la dernière décennie, de nouvelles lignées de parasites putatifs ont été révélées à partir d'échantillons d'hôtes et d'échantillons environnementaux (e.g Pagenkopp Lohan *et al.*, 2016; del Campo *et al.*, 2019; Muñoz-Gómez *et al.*, 2019). Globalement, les relations symbiotiques sont un vaste champ de découvertes dont seul un tiers des interactions est aujourd'hui élucidé chez les grands groupes d'eucaryotes (Figure 6).

2. Quand les parasites deviennent pathogènes

2.1. L'émergence de maladies infectieuses

Aujourd'hui, le changement climatique est largement reconnu comme étant un des principaux moteurs de la 6^{ème} extinction de masse de l'histoire de la Terre (Barnosky *et al.*, 2011). Pendant longtemps, l'intérêt scientifique s'est porté sur les conséquences affectant la distribution et l'abondance des grands taxa d'animaux et de plantes en occultant bien souvent l'impact occasionné sur leurs parasites. Les écosystèmes sont aujourd'hui menacés par de nombreux facteurs englobant la destruction des habitats, le changement climatique (e.g. dérèglement des

températures, augmentation du niveau de la mer), la pollution, l'introduction d'espèces exotiques la surexploitation ou encore les maladies (Pimm *et al.*, 1995; Wilcove *et al.*, 1998; Harvell, 2002). Les maladies sont une menace directe pour la biodiversité globale et notamment lorsque ces maladies sont des maladies infectieuses émergentes (aussi abrégées EID, « Emerging Infectious Disease »). Selon l'O.I.E, une EID désigne « une nouvelle apparition, chez un animal, d'une maladie, d'une infection ou d'une infestation ayant des répercussions significatives sur la santé animale ou humaine et résultant : **a**) de la modification d'un agent pathogène connu ou de sa propagation à une nouvelle aire géographique ou à une nouvelle espèce ; ou **b**) d'un agent pathogène non identifié antérieurement ou d'une maladie diagnostiquée pour la première fois » (O.I.E, 2019c). Les (micro-)organismes responsables de maladies occupent une place importante dans la liste des 100 espèces exotiques envahissantes parmi les plus néfastes au monde dressée par l'Union Internationale pour la Conservation de la Nature (UICN). Il s'agit du principal facteur de déclin des populations derrière les introductions d'espèces exotiques (Hatcher *et al.*, 2012). Ces maladies peuvent entraîner des événements de mortalité massive (EMM) impliquant dans la grande majorité des cas (26.3 %) des bactéries, des protistes, des virus et des champignons. Un exemple fort d'EIDs et d'EMM est représenté par le déclin mondial des populations d'amphibiens à cause de la dissémination sur tous les continents du parasite appartenant aux fungi, *Batrachochytrium dendrobatidis* (Daszak *et al.*, 2004; Hanselmann *et al.*, 2004; Fisher *et al.*, 2009). Ce parasite serait impliqué dans le déclin de 200 espèces sur les 350 infectés dont certaines, e.g. *Rheobatrachus* sp., *Atelopus zeteki*, *Taudactylus acutirostris*, sont totalement éteintes à ce jour (Fisher *et al.*, 2009).

2.2. Le cas des parasites exotiques et invasifs

Au cours des deux derniers siècles, le taux de première détection d'espèces exotiques a considérablement augmenté et 37 % des premières introductions recensées se sont déroulées ces 50 dernières années (1970-2014) (Seebens *et al.*, 2017). Être à la fois parasite et envahisseur peut représenter un cocktail explosif affectant directement ou indirectement les populations et les communautés de parasites et d'hôtes. En effet, l'absence d'histoire évolutive commune entre des hôtes indigènes et un parasite exotique invasif peut entraîner une forte vulnérabilité de ces populations, souvent naïves, face à cette nouvelle infection. Les effets négatifs engendrés sur ces espèces indigènes peuvent se répercuter en cascade à l'échelle des communautés et des

écosystèmes (Goedknecht *et al.*, 2016). Trois scénarii majeurs ont ainsi été répertoriés pour mieux comprendre le processus de colonisation de ces parasites invasifs (Figure 7) :

- 1) **Introduction par des stades infectieux libres** – Ce processus d’invasion est un phénomène courant chez les micro-parasites dans les écosystèmes marins. Les eaux de ballast en sont d’ailleurs un des principaux vecteurs (Cariton and Geller, 1993) viennent ensuite les sédiments, les eaux résiduelles et les biofilms (Drake *et al.*, 2007). Par exemple, le nombre de bactéries et de virus introduits dans la Baie de Chesapeake tous les ans est estimé à un ordre de grandeur de 10^{20} (Drake *et al.*, 2007). Concernant les protistes, Pagenkopp Lohan *et al.* (2017) ont mis en évidence une diversité insoupçonnée des protistes transportés via les eaux de ballastes dans trois ports américains. Au total, 8 561 OTUs de 35 taxa de protistes différents ont été identifiés dont des espèces toxiques comme *Gyrodinium* sp. ou des espèces potentiellement parasites appartenant aux Syndiniales et aux Perkinsozoa (Alveolata) (Pagenkopp Lohan *et al.*, 2017). Ces résultats confirment la règle des 10 % de Williamson selon laquelle il existe une forte probabilité d’importer un organisme dont des parasites dans un nouvel environnement.

- 2) **Introduction avec un hôte invasif sans « spillover »** - Les parasites peuvent être co-introduits avec un hôte étant lui-même introduit dans un nouvel environnement (Lymbery *et al.*, 2014). Dans certains cas, ces parasites co-introduits peuvent s’établir et se propager avec leur hôte d’origine sans phénomène de « spillover » (littéralement « débordement ») sur d’autres espèces d’hôtes indigènes du lieu d’introduction. Goedknecht *et al.* (2016) a répertorié 48 cas de parasites marins ayant été co-introduits avec son hôte dont 13 se montrent restreints à leur hôte d’origine dans la région d’introduction.

- 3) **Co-introduction et phénomène de « spillover »** - Contrairement au scénario 2, les parasites co-introduits peuvent infecter leur hôtes d’origine mais aussi d’autres espèces hôtes indigènes du lieu d’introduction (Kelly *et al.*, 2009). Goedknecht *et al.* (2016) ont estimé que ces « spillover » ont été signalés chez 35 espèces de parasites marins co-introduits dont 37 % sont des micro-parasites. Ces parasites capable d’étendre leur spectre d’hôte dans leur nouvelle zone d’introduction sont majoritairement introduits

fortuitement, ou non, par des vecteurs liés à l'aquaculture tels que les poissons (63 %), les mollusques (29 %) ou encore les crustacés (6 %) (Goedknecht *et al.*, 2016).

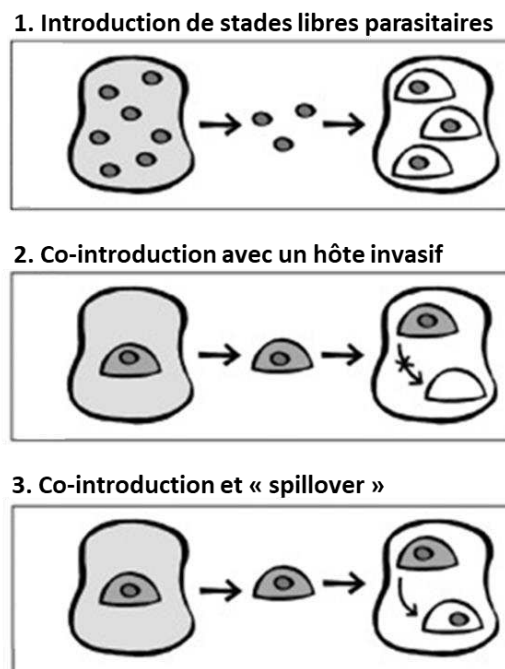


Figure 7. Trois scénarii représentant l'effet direct ou indirect d'un parasite invasif sur les interactions hôte-parasite d'après Goedknecht *et al.* (2016). Demi-cercle gris = espèce hôte invasive, demi-cercle blanc = espèce hôte indigène, point gris foncé = espèce parasite invasive, point blanc = espèce parasite indigène, environnement gris = aire de répartition de l'envahisseur dans le milieu d'origine, environnement blanc = aire de répartition de l'envahisseur dans le milieu d'introduction.

Ces trois scénarii peuvent conduire à de nouvelles émergences de maladie dont l'incidence (e.g la mortalité ou les effets sub-léthaux), la dispersion géographique et la manifestation peuvent être modifiées dans ces « nouveaux » écosystèmes (Daszak *et al.*, 2000; Goedknecht *et al.*, 2016).

Aujourd'hui, le taux du nombre d'espèces exotiques ne montre pas de signe de ralentissement à cause de politiques sanitaires peu efficaces et peu adaptées à nos modes de vie (Hulme, 2015; Seebens *et al.*, 2017). Le phénomène de dissémination de potentiel pathogène n'est donc pas prêt de s'arrêter. En effet, ces dernières années sont marquées par l'avènement de maladies comme la chytridiomycose chez les amphibiens (Scheele *et al.*, 2019) ou encore le morbilivirus chez les mammifères marins (Kennedy, 1998). L'étude de ces parasites est un défi scientifique

majeur dans un contexte d'émergence et d'augmentation de nouvelles zoonoses prenant de l'ampleur depuis les années 70 (Ward and Lafferty, 2004).

2.3. Intensification des EIDs : parasites généralistes ou spécialistes ?

L'intensification des EIDs peut être la conséquence de changement d'hôtes et de perturbations d'origine anthropique et/ou environnementale (Heffernan, 2018). Ces facteurs semblent favoriser l'émergence de parasites généralistes. En effet, Gog *et al.* (2002) ont modélisé les parasites ayant plusieurs hôtes alternatifs et/ou réservoirs comme des facteurs pouvant causer des déclin ou des extinctions de populations. Cet effet serait augmenté dans les cas où les populations seraient de faible effectif ou auraient une faible diversité génétique (Dobson and Foufopoulos, 2001; Castro and Bolker, 2005). Par exemple, de nombreuses espèces d'oomycètes, capables d'infecter des algues, des plantes, des protistes, des champignons, des arthropodes et des vertébrés (Kamoun, 2003), sont responsables de pertes massives dans l'agriculture, l'aquaculture et dans des populations sauvages (crustacés, amphibiens) (Phillips *et al.*, 2008). Chez les oiseaux, le parasite *Plasmodium relictum* (Apicomplexa, Alveolata) est responsable du déclin et de mortalités massives chez de nombreuses espèces d'oiseaux dans le monde (Valkiunas, 2004; Marzal *et al.*, 2015). Une tendance similaire est observée avec les parasites *B. dendrobatidis* et l'agent de la « Severe Perkinsea Infection » (SPI) (Perkinsozoa) responsables d'importantes mortalités chez les amphibiens avec de possibles extinctions d'espèces (Chambouvet *et al.*, 2020). Toutes ces observations soulèvent l'hypothèse qu'une part de la perte de la biodiversité globale pourrait être liée aux conséquences de l'augmentation des infections par des parasites généralistes à large spectre d'hôtes.

En effet, la présence de parasites spécialistes est fortement liée à celle de son hôte spécifique tandis que la survie des parasites généralistes peut être assurée par l'exploitation d'hôtes alternatifs (Poulin and Keeney, 2008). Cependant, cette dichotomie n'est pas immuable car certains parasites spécialistes conservent leur capacité à étendre leur spectre d'hôtes et inversement (un généraliste peut devenir spécialiste) (Agosta *et al.*, 2010). Malgré cette limite flexible, la sélection tend à favoriser la spécialisation (Poulin and Keeney, 2008). La spécialisation est le résultat d'une course aux armements menée entre l'hôte et son/ses parasites. Ce processus de coévolution menant à une baisse d'interactions avec d'autres hôtes potentiels

est favorisée dans des environnements peu fluctuants (Agosta *et al.*, 2010). Cependant, en général, les archives paléontologiques suggèrent que les espèces possédant une large répartition géographique ainsi qu'une forte capacité à se disséminer sont plus performantes pour survivre à des grands changements environnementaux et des épisodes d'extinctions de masse (Stigall and Lieberman, 2006). Ainsi les espèces généralistes représentent un véritable risque d'EID pouvant toucher tout type d'environnement (Agosta *et al.*, 2010). De plus, dans un contexte où les écosystèmes subissent de plus en plus fréquemment les introductions d'espèces et les effets des changements globaux, ces « sauts » vers de nouveaux hôtes s'avèrent être d'importants facteurs déclencheurs d'EIDs (Daszak *et al.*, 2000). Les changements d'hôtes comprennent deux événements : (1) l'expansion du spectre d'hôtes (colonisation) et, (2) la spécialisation (détachement de l'hôte ancestrale). Via ces deux processus, certains parasites peuvent se pérenniser dans de nouveaux écosystèmes au sein de nouvelles communautés (Woolhouse *et al.*, 2001; Font, 2003). Aujourd'hui, ces phénomènes de plus en plus fréquents à des échelles spatiales et temporelles restreintes conduisent à une véritable « mosaïque d'émergence » (Hoberg and Brooks, 2015). Aujourd'hui, de plus en plus de parasites généralistes sont identifiés mais les impacts sur les réseaux trophiques restent encore à déterminer comme c'est le cas pour les parasites de poissons comme *I. chabelardi* (Syndiniales), *Xcellia* spp. et *Gadixcellia* spp. (Perkinsozoa) (Gleason *et al.*, 2019; Freeman *et al.*, 2017), qui pourraient avoir un impact important sur les espèces pêchées de poissons.

3. « Un pathogène-une maladie », un concept obsolète dans l'étude des infections

Les infections multiples (infections simultanées avec plusieurs espèces de parasites chez un même hôte), également appelées polyparasitisme, sont la règle plutôt que l'exception dans la nature (Bordes and Morand, 2011). Les hôtes, y compris les animaux, les plantes ou les protistes, sont souvent co-infectés par plusieurs espèces de parasites (Petney and Andrews, 1998; Cox and Chappell, 2001). La polyinfection, ou infection concomitante, désigne un individu infecté simultanément par deux ou plusieurs espèces parasitaires. Ces assemblages de parasites montrent que le paradigme « un pathogène-une maladie » est dépassé et qu'il n'est pas suffisant pour expliquer l'évolution et les conséquences de certaines infections (Vayssier-Taussat *et al.*, 2014, 2015; Bass *et al.*, 2019). Lors d'études épidémiologiques, il est donc

important de considérer le maximum de parasites potentiels au sein d'un hôte infecté que de les traiter séparément. Cette nécessité a très vite été comprise dans le domaine médical et vétérinaire où de nombreuses études ont été réalisées aussi bien sur l'homme (par exemple, la co-infection du virus du SIDA et de l'agent du paludisme *Plasmodium falciparum* (Abu-Raddad *et al.*, 2006); de *Plasmodium falciparum* et d'helminthes (Brooker *et al.*, 2007); du virus du SIDA et la bactérie responsable de la tuberculose, *Mycobacterium tuberculosis* (Teweldemedhin *et al.*, 2018)) que sur la santé animale (par exemple, la polyinfection entre *Theileria parva*, *Trypanosoma* spp. et les strongyles chez les bovins (Thumbi *et al.*, 2014); plusieurs espèces de *Borrelia* chez les tiques (Raileanu *et al.*, 2017)). Ces enjeux sanitaires montrent qu'un diagnostic exact est fondamental pour contrôler la diffusion et les répercussions de possibles épizooties et zoonoses sur les populations hôtes et hôtes potentiels. Les infections multiples jouent sur la fitness et la sensibilité de l'hôte mais aussi sur la virulence et la transmission des parasites qui l'infectent (Pedersen and Fenton, 2007a). Ainsi, les conséquences de polyinfections peuvent être très variables et ne conduisent pas forcément à l'effet cumulé de chaque infection parasitaire prise séparément. En fonction des interactions au sein de ces communautés parasitaires hébergées par l'hôte, les polyinfections peuvent jouer en faveur de la fitness de l'hôte (effet protecteur), par exemple chez le monarque, *Danaus plexippus*, la co-infection entre le protiste parasite *Ophryocystis elektroscirrha* (Apicomplexa, Alveolata) et la mouche parasitoïde *Lespesia archippivora* (Diptera, Insecta) augmente les chances de survie de l'hôte (Sternberg *et al.*, 2011). Au contraire, elles peuvent être défavorables pour l'hôte, par exemple, la co-infection entre le virus du SIDA et la bactérie *Mycobacterium tuberculosis* (Actinobacteria) accélèrent le déclin des fonctions immunitaires (Pawlowski *et al.*, 2012a). En 2011, Bordes et Morand ont répertorié les impacts potentiels de la diversité des parasites et des infections multiples à l'échelle des individus et des populations montrant ainsi la complexité de leurs conséquences : augmentation de la variabilité des gènes MHC (« Major Histocompatibility Complex »), de l'hétérozygotie et de la résistance aux parasites contre une détérioration de l'indice de condition corporel, de la mobilité ainsi qu'une baisse de survie et de fécondité.

Les parasites peuvent interagir de plusieurs façons (Cox and Chappell, 2001; Graham, 2008) soit **directement**, par de la compétition où les mêmes niches trophiques et écologiques sont convoitées ; soit **indirectement**, par le système immunitaire de l'hôte. Bien souvent il est difficile de pouvoir trancher entre une compétition directe ou une médiation immunitaire comme c'est le cas pour la co-infection chez l'homme entre *Giardia lamblia* et des vers intestinaux (helminthes). Il a été montré que l'infection par des helminthes réduit la prévalence

à *G. lamblia*. Cette interaction antagoniste ne serait pas liée à une différence de sensibilité de l'hôte mais bien à une compétition directe ou à une médiation immunitaire par la production de cytokines Th2 (T-helper 2) fournissant une immunité contre *G. lamblia* (Blackwell *et al.*, 2013). Dans tous les cas, ces deux types d'interaction auront des effets négatifs sur le fitness et le développement d'un ou de plusieurs des parasites impliqués dans la co-infection.

La dynamique de polyinfection est un facteur clé pour comprendre l'issue de ces interactions car certains parasites peuvent tirer profit de l'ordre d'infection – primo-infection ou infection secondaire. Dans un premier temps, nous pouvons penser que l'infection séquentielle présente un grand inconvénient pour une espèce parasitaire qui rencontre un hôte déjà infecté, en raison de l'épuisement des ressources de l'hôte et de l'activation de son système immunitaire (Klemme *et al.*, 2016). Mais cette situation n'est pas toujours vraie, en effet, une première immunité acquise contre une première espèce pathogène peut avoir des effets positifs sur une deuxième espèce. En effet, l'affaiblissement du système immunitaire, peut conduire à une augmentation de la sensibilité à une infection secondaire (Montes *et al.*, 2001; Telfer *et al.*, 2008). Par exemple, une infection à *Perkinsus olseni* favorise les infection bactériennes et virales opportunistes en affaiblissant les défenses chez *Ruditapes philippinarum* (Montes *et al.*, 2001). Le parasitisme est un phénomène contexte-dépendant, où les cycles de vie, les dynamiques et les caractéristiques biologiques des hôtes et des parasites sont déterminants dans la manière dont s'expriment les polyinfections (Sternberg *et al.*, 2011). Il est maintenant urgent d'examiner ces cas d'infections multiples dans ce contexte d'EMM (Jones *et al.*, 2008) où la liste de détection des (nouveaux) agents pathogènes ne cesse de s'étendre. Il est clair que la polyinfection redistribue les cartes de nos connaissances acquises sur le modèle « un pathogène-une maladie » et c'est pour cela qu'il est important de mieux les comprendre afin de préserver des milieux déjà fragilisés par le changement global.

4. Le cas des micro-parasites de bivalves en milieu marin

4.1. Des écosystèmes côtiers essentiels et fragilisés

Les écosystèmes côtiers font partis des systèmes naturels les plus exploités et les plus menacés au monde car sujets à la dégradation, à la pollution, à la surexploitation, aux conséquences du changement climatique et à l'altération des fonctions écosystémiques (Harvell, 2002; Lotze *et*

al., 2006; Halpern *et al.*, 2008). Ces écosystèmes subissent une détérioration croissante face à ces pressions. Par exemple, 50 % des marais salants, 35 % des mangroves, 30 % des récifs coralliens et 29 % des herbiers marins ont disparu ou sont dégradés à travers le monde (proportions référencés dans Barbier *et al.*, 2011). La dégradation de ces systèmes entraîne un déclin des services écosystémiques dont l'Homme serait le plus grand perdant. Parmi les services écosystémiques les plus durement impactés, il est possible de répertorier : l'effondrement global des stocks de ressources naturelles, la diminution des zones de nurserie comme les récifs d'huîtres ou les herbiers marins, la baisse de la clairance et de la détoxification assurées par les organismes suspensivores ou les herbiers marins ou encore la diminution de la protection des côtes contre la houle et les tempêtes (Worm *et al.*, 2006; Koch *et al.*, 2009; Barbier *et al.*, 2011). D'un point de vue écologique et économique, les services écosystémiques rendus par les zones côtières sont primordiaux pour l'Homme et nécessitent une surveillance plus accrue afin de limiter leur déclin (Ruckelshaus *et al.*, 2013). Globalement, l'augmentation du trafic de ressources vivantes pour l'aquaculture et la consommation humaine ainsi que la perte de biodiversité, d'habitat et de fonctionnalité dans les écosystèmes façonnent un terrain propice pour les invasions biologiques concernant aussi bien les hôtes que leurs parasites. Ces invasions, comme décrites précédemment, entraînent des déséquilibres dans les relations hôte-parasites indigènes. Les facteurs de stress évoqués ci-dessus peuvent participer à l'expansion de certains parasites et/ou de leur vecteur dans de nouvelles niches écologiques pouvant entraîner d'importantes mortalités dans de nouvelles aires géographiques selon «the Enemy Release Hypothesis (ERH)» (Torchin and Lafferty, 2009). Ces introductions de parasites peuvent engendrer une déstabilisation des réseaux trophiques (Combes, 1996). Parmi les vecteurs introduits, les espèces les plus fréquemment détectées font partie des crustacés, des mollusques et des poissons comprenant aussi leurs parasites associés (Blakeslee *et al.*, 2013). Par exemple, le parasite *Haplosporidium nelsoni* est un cas de protiste ayant causé des mortalités massives chez les huîtres en baie de Delaware et de Chesapeake (Etats-Unis). Ce parasite a été introduit aux Etats-Unis après l'importation d'huîtres infectées en provenance du Japon (Barber *et al.*, 1997). Un cas similaire a été observé dans les années 70 en Europe avec des mortalités occasionnées par le protiste *Bonamia ostreae* importé des Etats-Unis (Peeler *et al.*, 2011). Ces infections peuvent présenter d'importantes pertes économiques et écosystémiques à cause d'un fort risque d'extinction chez les populations les plus vulnérables au phénomène de débordement par les parasites introduits (Gaino *et al.*, 1992; Pedersen *et al.*, 2007b; Scheele *et al.*, 2019).

4.2. La diversité micro-parasitaire infectant les organismes benthiques et démersaux

Dans le compartiment benthique, les organismes peuvent aussi bien être infectés par des parasites présents dans un hôte vecteur, dans la colonne d'eau ou dans les sédiments. En effet, certains parasites forment des stades de résistance pouvant contaminer durablement le sédiment, comme cela a été démontré expérimentalement chez *Perkinsus olseni* (Park *et al.*, 2010). Contrairement aux écosystèmes pélagiques, il est plus difficile d'appréhender la dynamique et le rôle écologique de ces parasites micro-parasites eucaryotes. Des pistes pointent aussi leur rôle d'ingénieur des écosystèmes en influençant l'activité d'épuration et de bioturbation (Dairain *et al.*, 2019; Williams *et al.*, 2019) qui se trouve en lien direct avec le cycle de certains éléments comme le fer (Simão *et al.*, 2010; Vannatta and Minchella, 2018). Finalement, l'intérêt économique est le plus souvent privilégié focalisant les études sur la gestion des stocks, la réponse immunitaire des hôtes face aux parasites ou encore la recherche de molécules anti-protistes. Les micro-parasites eucaryotes étudiés sont majoritairement affiliés à des espèces de crustacés, de poissons et de mollusques présentant un intérêt économique pour l'Homme. Selon le rapport (FAO, 2016), en 2014, la production totale de la pêche de capture marine a été estimée à 81.5 millions de tonnes tandis que la production de l'aquaculture s'est établie à 73.8 millions de tonnes, pour une valeur estimée à 160.2 milliards de dollars, dont 49.8 millions de tonnes de poissons, 16.1 millions de tonnes de mollusques, 6.9 millions de tonnes de crustacés. Deux infra-règnes sont bien souvent responsables des épidémies menaçant ces ressources marines :

- (1) **les Alveolata** (Figure 8): Chez les crustacés, le parasite le plus emblématique est le dinoflagellé *Hematodinium* qui infecte des organismes à forte plus-value tels que les langoustines, *Nephrops norvegicus*, ou encore les tourteaux, *Cancer pagurus* (Stentiford and Shields, 2005). En Ecosse, la pêche à la langoustine a subi de lourdes pertes dues à ce parasite dans les années 90 (Field *et al.*, 1992). D'autres phénomènes épidémiques liés à ce parasite ont été reportés dans les populations de *Chionoecetes opilio* et *C. bairdi* (crabe des neiges), à Terre-Neuve et en Alaska (Stentiford and Shields, 2005; Shields *et al.*, 2007). Pour les poissons, les micro-parasites les plus fréquents sont les dinoflagellés *Amyloodinium ocellatum* et *Ichthyodinium chabelardi* (Skovgaard *et al.*, 2009; Gleason *et al.*, 2019) ainsi que les Perkinsozoa appartenant au groupe des Xcellidae (Freeman *et al.*, 2017). *Amyloodinium* est considéré comme la plus

sérieuse des menaces pour la pisciculture; jusqu'à 100 espèces de poissons différentes y sont susceptibles, englobant les Elasmobranches et les Téléostéens (Woo and Leatherland, 2006). Chez les mollusques, le genre *Perkinsus* (Perkinsozoa) regroupe des agents pathogènes redoutables dont les spectres d'hôtes touchent aussi bien les gastéropodes que les bivalves. Les menaces les plus importantes sont représentées par les deux espèces, *Perkinsus marinus* et *Perkinsus olseni*, qui figurent parmi les pathogènes à surveiller dans le Code de la Santé des Animaux Marins établis par l'Organisation Mondiale pour la Santé Animale (O.I.E, 2019a).

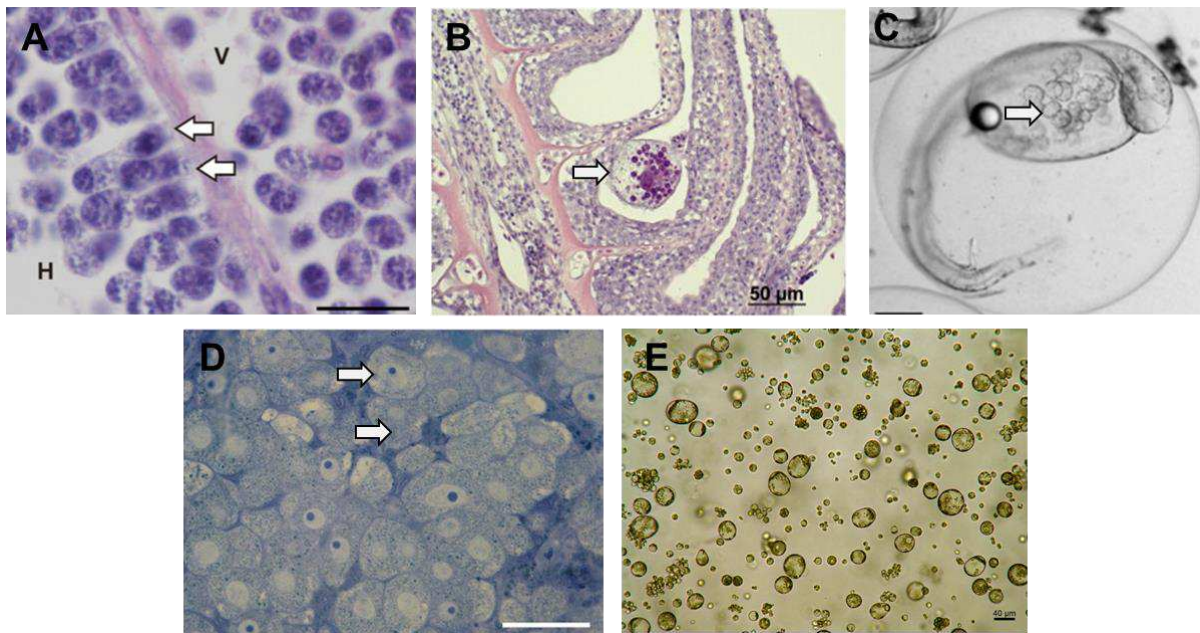


Figure 8. Micro-parasites eucaryotes appartenant aux Alveolata. (A) *Hematodinium perezii* infectant *Liocarcinus depurator*. Les trophonts (flèches blanches) sont attachés à la paroi du vaisseau transportant l'hémolymphe (V) (Small *et al.*, 2012). (B) *Amyloodinium ocellatum* dans les filaments branchiaux du turbot (Saraiva *et al.* 2011). (C) Œufs d'anguilles infectés par *Ichthyodinium chabelardi* (Skovgaard *et al.* 2009). (D) Section d'un xénome contenant des cellules de *Gadixcellia gadi* (Freeman *et al.* 2017). (E) Trophozoites de *Perkinsus chesapeaki* en culture. Echelles de taille : (A) 25 μm , (B) 50 μm , (C) 100 μm , (D) 10 μm , (E) 40 μm .

(2) **les Rhizaria** (Figure 9): Les genre *Haplosporidium*, *Marteilia* et *Bonamia* sont à l'origine d'importants phénomènes de mortalité chez certaines espèces de mollusques exploités à travers le monde. A ce jour, des parasites comme *Haplosporidium nelsoni*, *Marteilia refringens* et *Bonamia ostreae* sont donc sous la surveillance de l'Organisation

mondiale de la santé animale (O.I.E, 2019c,a). *H. nelsoni*, agent étiologique de la Multinucleated Sphere X (MSX), a été responsable du déclin de 90 à 95 % des huîtres en baie du Delaware (États-Unis) dans les années 50 (Ford and Haskin, 1982) . *M. refringens* et *B. ostreae* ont été associées à des mortalités chez l'huître plate européenne, *Ostrea edulis*, en Bretagne dans les années 70 (Comps, 1970; Grizel *et al.*, 1974; Pichot *et al.*, 1979). Ces évènements de mortalités sont liés à l'introduction de ces parasites dans des environnements où ils n'avaient jamais été détectés auparavant (Grizel *et al.*, 1988; Burreson *et al.*, 2000).

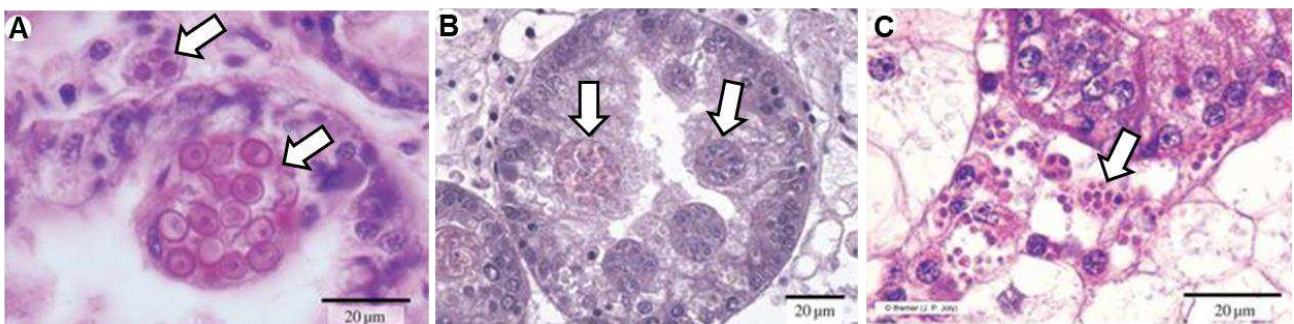


Figure 9. Micro-parasites eucaryotes appartenant aux Rhizaria tiré de Carnegie *et al.* (2016). (A) *H. nelsoni* infectant *C. virginica*. (B) *Marteilia refringens* infectant *Ostrea edulis*. (C) *Bonamia ostreae* infectant *Ostrea edulis*.

4.3. Les Perkinsozoa : un groupe clé de parasites généralistes

Les Perkinsozoa (syn. Perkinsea, Perkinsides) sont un groupe de micro-parasites eucaryotes généralistes à large spectre d'hôtes appartenant au phylum des Alveolata. Certains de ses membres ont été étiquetés comme menaces écologiques et économiques majeures en raison de leur capacité d'invasion dans de nouvelles aires géographiques, de leur pathogénicité (évènements de mortalité et effets sub-léthaux) et de leur grande plasticité phénotypique. Dans ce groupe, l'un des exemples les plus célèbres de maladies émergentes est la maladie "Dermo" causée par le protiste, *Perkinsus marinus* (Ray, 1952). Ce parasite a été identifié comme responsable de mortalités massives d'huîtres américaines (*Crassostrea virginica*), qui ont entraîné l'effondrement des industries ostréicoles dans le golfe du Mexique et la baie de Chesapeake (États-Unis) (Burreson *et al.*, 1994; Andrews, 1996). Bien que la première détection remonte aux années 50, les Perkinsozoa ont souvent été sous-estimés et sous-

échantillonnés dans les écosystèmes aquatiques, à l'exception des agents infectieux, *P. olseni* et *P. marinus*. Mais aujourd'hui, grâce aux avancées dans le domaine de la biologie moléculaire et à l'intérêt grandissant pour les problématiques portant sur l'augmentation des maladies infectieuses à travers le monde, quatre grands groupes de Perkinsozoa ont été décrits dans une grande variété d'écosystèmes aquatiques: les Parviluciferaceae - parasites de dinoflagellés (Norén *et al.*, 1999), les Perkinsidae – parasites de mollusques incluant bivalves et gastéropodes (Mackin *et al.*, 1950 ; Reece *et al.*, 1997), les Xcellidae – parasites de poissons (Freeman *et al.*, 2017) et enfin l'agent de la « Severe Perkinsea Infection » (SPI) (ou Novel Alveolate Group 01, NAG01) – parasites d'amphibiens (Davis *et al.*, 2007; Chambouvet *et al.*, 2015; Isidoro-Ayza *et al.*, 2017). De nombreuses études environnementales ont révélé que les Perkinsozoa étaient très diversifiés et abondants notamment dans les écosystèmes d'eau douce où ils jouent un rôle écologique important dans la dynamique des réseaux trophiques (Lepère *et al.*, 2008; Mangot *et al.*, 2011; Jobard *et al.*, 2020). En revanche, ils se font plus rares mais tout aussi diversifiés dans les milieux marins où leurs abondances relatives sont souvent inférieures à 1 % du jeu de données (Chambouvet *et al.*, 2014; De Vargas *et al.*, 2015). A la vue des caractéristiques de ce groupe, la présence de ces signatures génétiques interroge sur le rôle fonctionnel qu'ils occupent dans les écosystèmes ainsi que sur leur potentiel à être eux-mêmes des parasites.

5. Objectifs et déroulement du projet de thèse

Cette thèse se concentre sur le groupe des Perkinsozoa (Alveolata) dont tous les organismes déjà décrits sont parasites et occupent une place primordiale au sein des écosystèmes aquatiques. Ses représentants emblématiques sont les parasites appartenant au genre *Perkinsus*, plus spécifiquement *Perkinsus olseni* et *Perkinsus marinus*, ayant causé d'importantes mortalités à travers le monde chez les populations de palourdes et d'huîtres respectivement. A ce jour, *Perkinsus olseni* et *Perkinsus marinus* font l'objet d'une surveillance accrue par des réseaux internationaux, comme l'Organisation mondiale pour la Santé Animale ou encore la Commission Européenne (Directive 2006/088/EC). Ces réseaux sont aujourd'hui essentiels dans la gestion des ressources aquacoles et dans la gestion de la dynamique de dissémination de ces parasites.

Récemment, grâce au séquençage nouvelle génération, de nombreuses études environnementales ont relevé que les Perkinsozoa étaient un groupe beaucoup plus diversifié

que les quatre clades déjà décrits. De nombreux clades, en marin comme en eau douce, restent simplement décrits par leur signature génétique. Même si la majorité des Perkinsozoa déjà décrits est parasite, le rôle fonctionnel de cette diversité environnementale reste énigmatique et nécessitera de nombreux efforts scientifiques afin d'identifier ces organismes et leur réseau d'interaction.

Dans un contexte d'augmentation des EIDs, les caractéristiques dévastatrices affiliées aux Perkinsozoa et à leur potentiel diversité environnementale pourraient poser de nouvelles problématiques sanitaires et sociétales. Dans cette thèse, nous aborderons l'aspect généraliste des Perkinsozoa et nous nous pencherons sur leur diversité environnementale retrouvée dans la colonne d'eau à l'échelle de l'océan mondiale. Puis, nous étudierons plus en détail la relation entre *Perkinsus olseni* et *Perkinsus chesapeaki*, deux parasites présents sur la façade atlantique française. En effet, la Perkinsose cache parfois des associations plus complexes. Connaître la diversité parasitaire au sein d'un hôte, comprendre sa répartition et son fonctionnement est un point absolument essentiel pour la gestion des épizooties en milieu marin. Ainsi, ce travail doctoral s'articule en deux grands axes :

- (1) **Les Perkinsozoa** : un groupe parasitaire généraliste disséminé à travers le monde.
 - **Chapitre 1.1** : Une nouvelle ère des parasites émergents : les Perkinsozoa sont-ils une menace ? [Draft review - soumission prévue fin 2020]
 - **Chapitre 1.2** : Signatures génétiques des Perkinsozoa au sein des échantillons de la campagne Malaspina 2010 Circumglobal Expedition : une diversité environnementale insoupçonnée basée sur la région V4 de l'ARNr 18S. [Draft court rapport]

- (2) **Cas de *Perkinsus*** : Détection et distribution de la co-infection entre deux espèces de *Perkinsus* à l'échelle locale et individuelle.
 - **Chapitre 2.1** : Développement d'une méthode moléculaire basée sur la PCR quantitative en temps réel TaqMan pour la détection simultanée de *Perkinsus olseni* et *P. chesapeaki* chez les palourdes japonaises, *Ruditapes philippinarum*, du bassin d'Arcachon. [Publication - soumis en Novembre 2020 dans Journal of Invertebrate Pathology]
 - **Chapitre 2.2** : Occurrence et distribution de deux parasites invasifs émergents, *Perkinsus olseni* et *P. chesapeaki*, de l'échelle individuelle à l'échelle locale (bassin d'Arcachon, France). [Draft publication - soumission prévue en 2021]

- **Chapitre 2.3 :** Génomes de deux parasites, *P. olseni* et *P. chesapeakei* (Perkinsozoa), co-infectant la palourde japonaise, *Ruditapes philippinarum*, dans le bassin d'Arcachon. [Draft d'annonce du séquençage des génomes de *P. olseni* et *P. chesapeakei*]

La première partie fait état des connaissances globales sur les Perkinsozoa : leurs caractéristiques, leur cycle de vie, leur spectre d'hôtes, leur stratégie parasitaire et leurs potentiels effets délétères sur la biodiversité et les activités humaines. Ce groupe encore peu connu a été détecté au sein du nanoplancton dans différents océans (0.2 – 2 µm) échantillonnés lors de la campagne Malaspina 2010. Ces représentants sont uniquement composés de nouvelles signatures génétiques basées sur leur séquence V4 de l'ADNr 18S dont l'activité ribosomale est active. Cette diversité n'est qu'un premier pas pour comprendre le rôle de ces relations symbiotiques au sein du réseaux microbiens marins.

La deuxième partie s'articule autour d'un cas d'étude portant sur la co-infection entre deux espèces appartenant au genre *Perkinsus*. Nous avons détecté la co-occurrence de *P. olseni* et *P. chesapeakei* dans les palourdes japonaises au sein du bassin d'Arcachon alors que seul *P. olseni* avait été identifié jusqu'ici. Pour l'étude de cet assemblage entre deux espèces de *Perkinsus*, nous avons mis en place une méthodologie de PCR quantitative en temps réelle en duplex afin de détecter et quantifier ces deux parasites à travers les différents organes de leurs hôtes. La répartition de la co-infection a été prospectée à l'échelle du bassin et à l'échelle des individus afin de déterminer si certains facteurs biotiques ou abiotiques pouvaient influencer leur répartition. Enfin, les génomes de ces deux parasites ont été séquencés à partir de souches isolées dans le bassin, dans le but de mieux comprendre les mécanismes de virulence, de prolifération et d'infection spécifiques à chacune des deux espèces.

PARTIE 1

Les Perkinsozoa : un groupe de parasites généralistes disséminés à travers le monde

Chapitre 1.1 : Une nouvelle ère de micro-parasites émergents : le cas de Perkinsozoa ?

Avant-propos

Au cours de ce dernier siècle, le changement global a profondément altéré l'équilibre des relations hôtes-parasites, entraînant une perte permanente de la biodiversité et favorisant l'émergence de nouvelles maladies infectieuses à travers le monde. Au cours de ce **Chapitre 1.1**, nous avons fait état des connaissances sur les Perkinsozoa, un phylum composé exclusivement de micro-parasites eucaryotes parasites aux larges spectres d'hôtes. Certains de ces parasites représentent de réelles menaces écologiques et économiques en raison de leur caractère invasif, de leur pathogénicité (conduisant à des cas de mortalité) et de leur grande plasticité phénotypique. Dans l'environnement marin, trois groupes sont actuellement décrits : les Perkinsidae, les Parviluciferaceae et les Xcellidae, qui infectent respectivement les mollusques, les dinoflagellés et les poissons. En revanche, un seul groupe est officiellement décrit en milieu d'eau douce : l'agent de « Severe Infection Perkinsea », infectant les amphibiens. L'avènement des NGS a permis la multiplication des études de diversité environnementale majoritairement basées sur l'analyse de l'ARNr 18S. Cette technologie a permis de révéler, en milieu dulcicole ou marin, que les Perkinsozoa étaient beaucoup plus diversifiés que les quatre lignées précédemment décrites. La découverte de cette « black box » microbienne présente de nouvelles perspectives de recherche pour comprendre la place de ces organismes relatifs au Perkinsozoa dans les écosystèmes et pour prévenir d'éventuels impacts négatifs (e.g introduction d'espèce, agrandissement du spectre d'hôte). Ce **chapitre 1.1** résume donc les caractéristiques biologiques et écologiques des Perkinsozoa incluant leur diversité, leur cycle de vie, leurs préférences d'hôtes, leur pathogénicité et leur distribution afin de mieux comprendre leur(s) rôle(s) écologique(s) et fonctionnel(s) à l'échelle globale.

A new era of emerging parasitic protists: the case of Perkinsozoa?

Abstract

The last century has witnessed an increasing rate of new disease emergence across the world. Global change has deeply disrupted host-parasite equilibria, leading to permanent loss of biodiversity. Perkinsozoa is a micro-eukaryotic parasitic phylum dominated by generalist parasites with broad host ranges. Some Perkinsozoa represent major ecological and economical threats because of their geographically invasive ability, pathogenicity (leading to mortality event) and high phenotypic plasticity. In the marine environment, the three families Parviluciferaceae, Perkinsidae and Xcellidae, infecting respectively dinoflagellates, molluscs and fish, are currently described. In contrast, only one group is officially described in freshwater environment: the agent of Severe Perkinsea Infection agent, infecting frog tadpoles. The advent of high throughput sequencing methods, mainly based on 18S rRNA assays, showed Perkinsozoa to be far more diverse than the previously four lineage described. The unveiling of this microbial black box presents a multitude of research challenges to understand their ecological roles and ultimately to prevent their most negative impacts. This review summarises the biological and ecological traits of Perkinsozoa - their diversity, life cycle, host preferences, pathogenicity, and highlights their diversity and ubiquity in association with a wide range of hosts.

1. Introduction

Parasitism is a key component in all ecosystems, playing a fundamental role at population level and wider ecological scales. Although parasites play a key role in food web interactions (Bjorbækmo *et al.*, 2020), their diversity, dynamic and influence on ecosystems remain neglected (Lafferty *et al.*, 2006). In marine ecosystems, global environmental sequencing studies, based on the analysis of the small subunit rRNA encoding gene (SSU rDNA) have revolutionized our conception of the microbial food webs with the (re-)discovery of an undescribed diversity shaping a more complex and cryptic global ocean interactive network (Lima-Mendez *et al.*, 2015; Guidi *et al.*, 2016). However, the vast majority of these putative parasitic organisms are still today only identified by taxonomic marker gene sequences. Fundamental questions about host preference, range, and interactions, remain unanswered.

Parasitic organisms are defined on the basis of their trophic characteristics and therefore by their hosts range which allow or not the spread to a novel host species or/and into new biogeographical areas (Cleaveland *et al.*, 2001; Poulin and Mouillot, 2003). The host range, defined by Lymbery (1989), considers the number of host species by a given parasite species. Two main categories can be distinguished by the extent diversity of hosts infected: the narrow host range (NHR) and the broad host range (BHR). Despite NHR parasites seems slightly to dominate the host-symbionts networks as described by Bjorbækmo *et al.* in 2020, this dichotomy between BHR and NHR parasites is not static and events like host shift can lead NHR to become BHR parasites and vice versa (Agosta *et al.*, 2010). Although the distinction between NHR and BHR seems to be clear-cut for some parasitic taxa, it is not the case for others. Hence, Desdevises *et al.* (2002) developed the Non-Specific Index (NSI) which classifies parasites based on their host range and the relatedness between host organisms. This index was adapted by Šimková *et al.* (2006) as the Specificity Index (SI) based on cyprinid fish and *Dactylogyrus* spp. (monogenean gill flukes) model with the following categories: (1) strict specialist living on a single host species, (2) intermediate specialist living on two or more host species from the same genus, (3) intermediate generalist living on two or more non-congeneric species of the same terminal clade, (4) generalist living on different hosts belonging to one hosts family and (5) “true” generalist living on different host species of different families (Figure 1A).

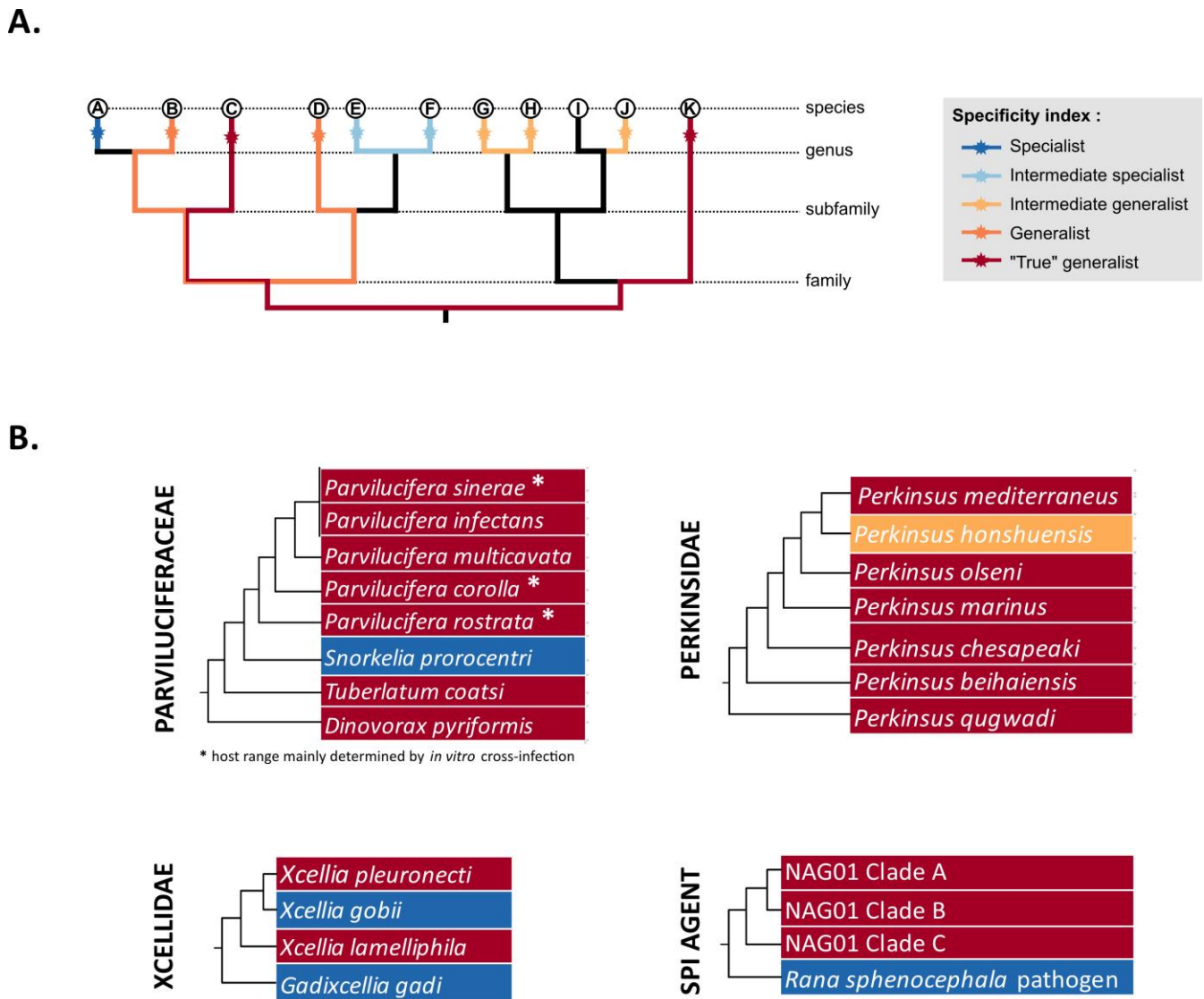


Figure 1. Specificity index within the Perkinsozoa members. **A)** Class of specificity index adapted from Desdevises *et al.* (2002) and Šimková *et al.* (2006). Specialists and intermediate specialists are considered as narrow host range (NHR) whereas intermediate generalists, generalists and “true” generalists are considered as broad host range (BHR). **B)** Schematic phylogenies of Parviluciferaceae, Perkinsidae, Xcellidae and “Severe Perkinsea Infection” (SPI) agent where each species was affiliated to a specificity index class. Colours on phylogenies corresponds to colour code of the specificity index.

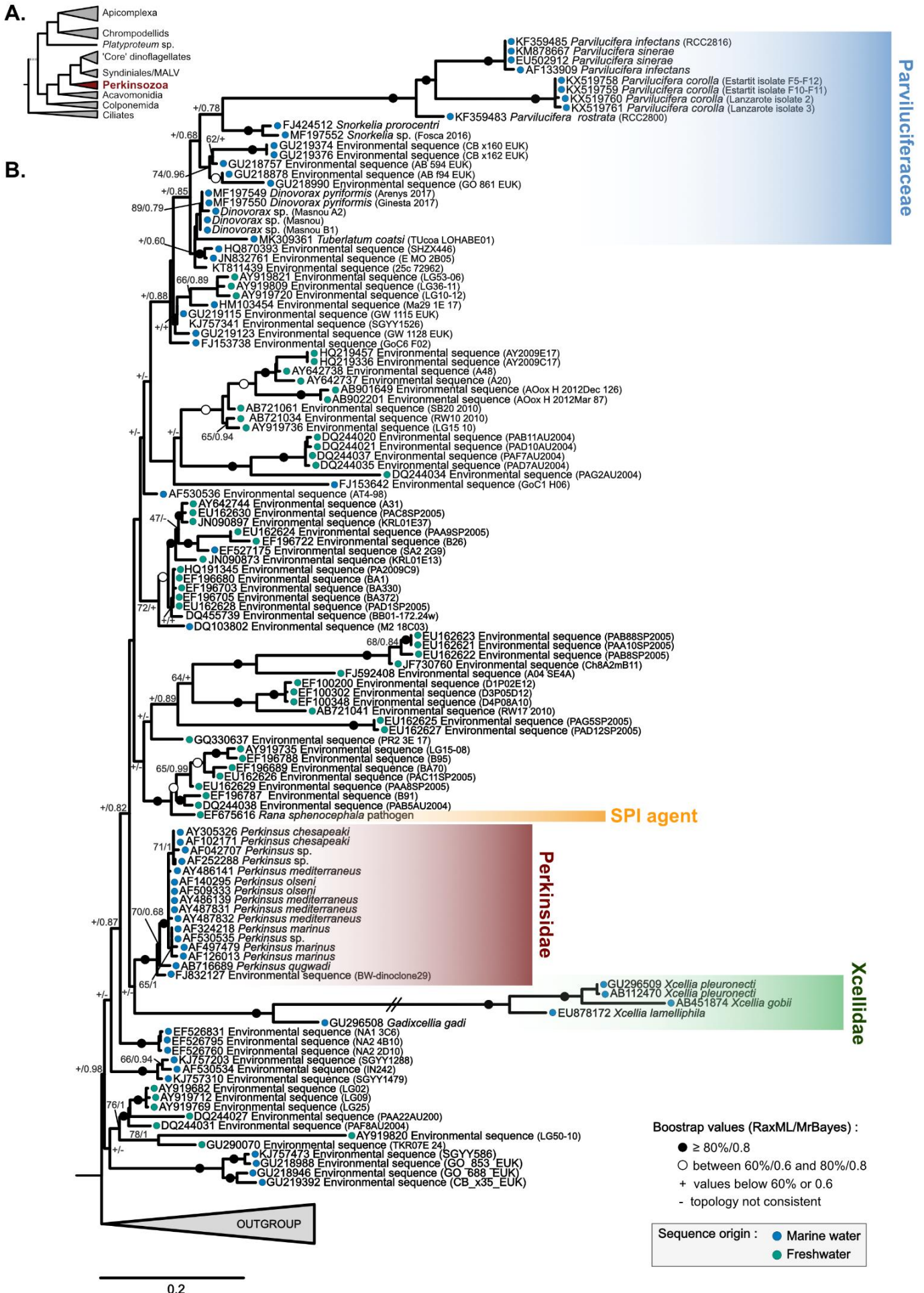
However all these indexes do not take into account the ecological fitting (=phenotypic flexibility and phylogenetic conservatism in traits related to resource use), or the parasite’s preferences or the availability of potential hosts which are essential to establish *in situ* host range of parasitic species (Agosta *et al.*, 2010). Considering only the phylogenetic level in a host range can lead to misinterpretation and a part of arbitrary decision will always last in the classification of generalist and specialist parasites. In this review, we consider the host range in terms of species richness following the SI but also in terms of the scientific knowledge accumulated on these host-parasite interactions. We choose to define BHR parasite as an

organism able to ensure its own survival through the colonization of divergent host species in a same ecosystem or following a translocation event.

Roles and impacts of parasitic protists harboring various host range are fundamentally different on ecosystems functioning. At ecosystem-scale, NHR parasites induce a structuration of populations by facilitating species coexistence and thus a maintenance of biodiversity (Janzen 1970; Connell 1978; Hudson *et al.*, 2006). This phenomenon has been observed in Penzé estuary (France) where eukaryotic parasites (MALV II, Alveolata) specifically infect four dinoflagellates species leading to a specific temporal succession (Montagnes *et al.*, 2008; Chambouvet *et al.*, 2008). Conversely, BHR parasites, through a difference in host susceptibility, can influence inter-specific competitiveness by disrupting the structuring of host populations and thus potentially reducing biodiversity (Hudson *et al.*, 2006). In 2002, Kassen suggested that parasites with restricted host spectra are likely favored during periods of environmental stability (or homogeneity) while those with larger host spectra are likely favored during instability (or heterogeneity).

In recent years, this equilibrium between hosts and parasites has been modified by anthropogenic activities (e.g. global warming, habitat degradation, mass extinction and introduction of exotic host and/or parasitic species). This phenomenon is illustrated by increases in prevalence and severity of infectious epidemics which can lead to mortality events (Harvell, 1999; Daszak, 2000). Indeed, interactions between a host and its pathogens are not fixed in time and space. They depend both on the quality and efficiency of host's immune defense against pathogens and on mechanisms used by parasites to bypass these defense systems to establish infection (Råberg *et al.* 2014). Studies on metazoan host-protist parasite model provide evidence that environmental stressors, e.g. temperature, pollution, are known to alter the immune system and make hosts more susceptible to parasites (Morley, 2010; Goedken *et al.*, 2005). Conversely, studies on environmental stressors modifying protist host-protist parasite relationship are still undiscovered but few studies revealed that, for example, herbicide exposure could lead of an increase of chytrid infections in phytoplankton populations (e.g. Van den Wyngaert *et al.*, 2013). Hence, many studies targeting metazoan host populations have recently highlighted the impact of climate change on terrestrial parasites dissemination (Daszak, 2000) and, to a lesser extent, of marine parasites dissemination (see Burge *et al.* 2014).

Chapitre 1.1 : Une nouvelle ère des micro-parasites émergents : les cas des Perkinsozoa ?



◀ **Figure 2.** Major phylogenetic relationships within the Perkinsozoa and others Alveolates. A) Schematic radiation of Alveolata superphylum (not to scale) based on their rDNA phylogeny adapted from Chambouvet *et al.* 2020. The basal branch (dotted line) is hypothetical. B) RAxML tree investigating the Perkinsozoa diversity based on 18S rRNA. The phylogeny was calculated from 135 taxa and 728 character alignment positions. ML bootstrap values (1 000 replicates) and Bayesian posterior probability (2 000 000 generations, GTR+ Γ +I model) were notated using the following convention: support values are summarized by black circles when $\geq 80\%/0.8$ and white circle when it is not the case but values $\geq 60\%/0.6$. When support value is $< 60\%/0.6$ but topology is consistent, the support value is summarized by a '+' and conversely by a '-' if the topology is not consistent. The sequence origins are represented by blue circle for marine water and green circle for freshwater. Thirteen sequences of dinoflagellates, syndiniales and MALV (Marine ALVeolate) were used as an outgroup. *Parvilucifera multicavata* is not included in the phylogenetic tree because the 18S rDNA sequence is not available on Genbank database.

In addition, introduction of exotic species, described as a global phenomenon, is also considered a major threat to global biodiversity (Cohen, 1998; Galil, 2000; Goedknecht *et al.*, 2016). Over the last two centuries, 37% of the first recorded species introduction phenomenon occurred in the last 50 years (1970-2014) and this trend is not slowing down (Seebens *et al.*, 2017). In aquatic ecosystems, the unintentional introduction of exotic species has increased substantially with the globalization of economies: *Haplosporidium nelsoni* (hypothetic living-stock vector: *Crassostrea gigas*) translocated from Asia to the United States (U.S.A.) (Burrison *et al.*, 2000) or *Bonamia ostreae* (hypothetic living-stock vector: *Ostrea edulis*) translocated from U.S.A. to Europe (Elston, 1986). Each day, hundreds of species are passively transferred across the oceans by sea transport (e.g. ballast water) and / or stock exchange of organisms (Ruiz *et al.*, 2000). If the majority of introductions fail due to the hostility of the new ecosystem (de Montaudouin *et al.* 2001; Williamson & Fitter 1996), some find a new niche for their development and reproduction (e.g. the absence of enemies such as predators, parasites, competitors). Moreover, parasitic micro-organisms with a high potential to invade and quickly adapt to a new environment share several common traits as being *r*-strategist, a wide host range with high phenotypic plasticity, high dispersal capacities and genetic diversity (Sakai *et al.*, 2001; Litchman, 2010). All these conditions form the "Ecological roulette" described by

Cariton & Geller (1993). In the case of introduction of parasites, the emergence of new pathologies might lead to disastrous consequences for the host populations.

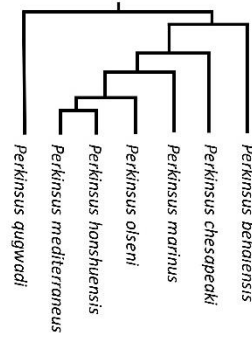
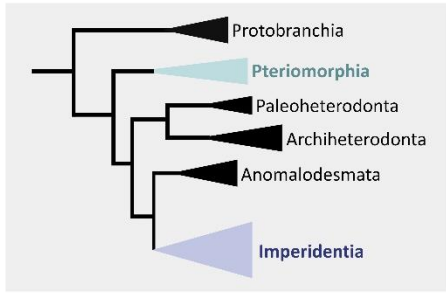
One of the most famous examples of an emerging disease is the "Dermo" disease caused by the virulent and invasive protist *Perkinsus marinus* (Perkinsozoa, Alveolata). This parasitic protist has been identified as responsible for the mass mortalities of oysters (*Crassostrea virginica*), driving the oyster industry to collapse in the Gulf of Mexico and the Chesapeake Bay (USA) (Burreson *et al.*, 1994; Andrews, 1996). This micro-eukaryote belongs to the Perkinsozoa (syn. Perkinsids, Perkinsea) lineage Alveolata, which has long been ignored in aquatic ecosystems with the exception of the notifiable agents, *P. olseni* and *P. marinus*. Nowadays, four parasitic groups of parasitic Perkinsozoa have been described from a wide variety of aquatic ecosystems: Parviluciferaceae, Perkinsidae, Xcellidae and the Severe Perkinsea Infection (SPI) agent (or Novel Alveolate Group 01, NAG01) (Figure 2). Surprisingly, recent studies revealed that the Perkinsozoa host range is broader than previously thought from micro-algae, molluscs, fishes to tadpoles. Due to a general lack of knowledge of the biology and diversity of these micro-parasites, our understanding of their impact on the structure and functioning of aquatic ecosystems remains limited. Our objective here is to review the main discovery of this parasitic lineage whose representative organisms harbour a BHR, strong phenotypic plasticity and are therefore candidates to be successful potential invasive species in a context of global change.

2. *Perkinsus* spp., the emerging parasite of molluscs

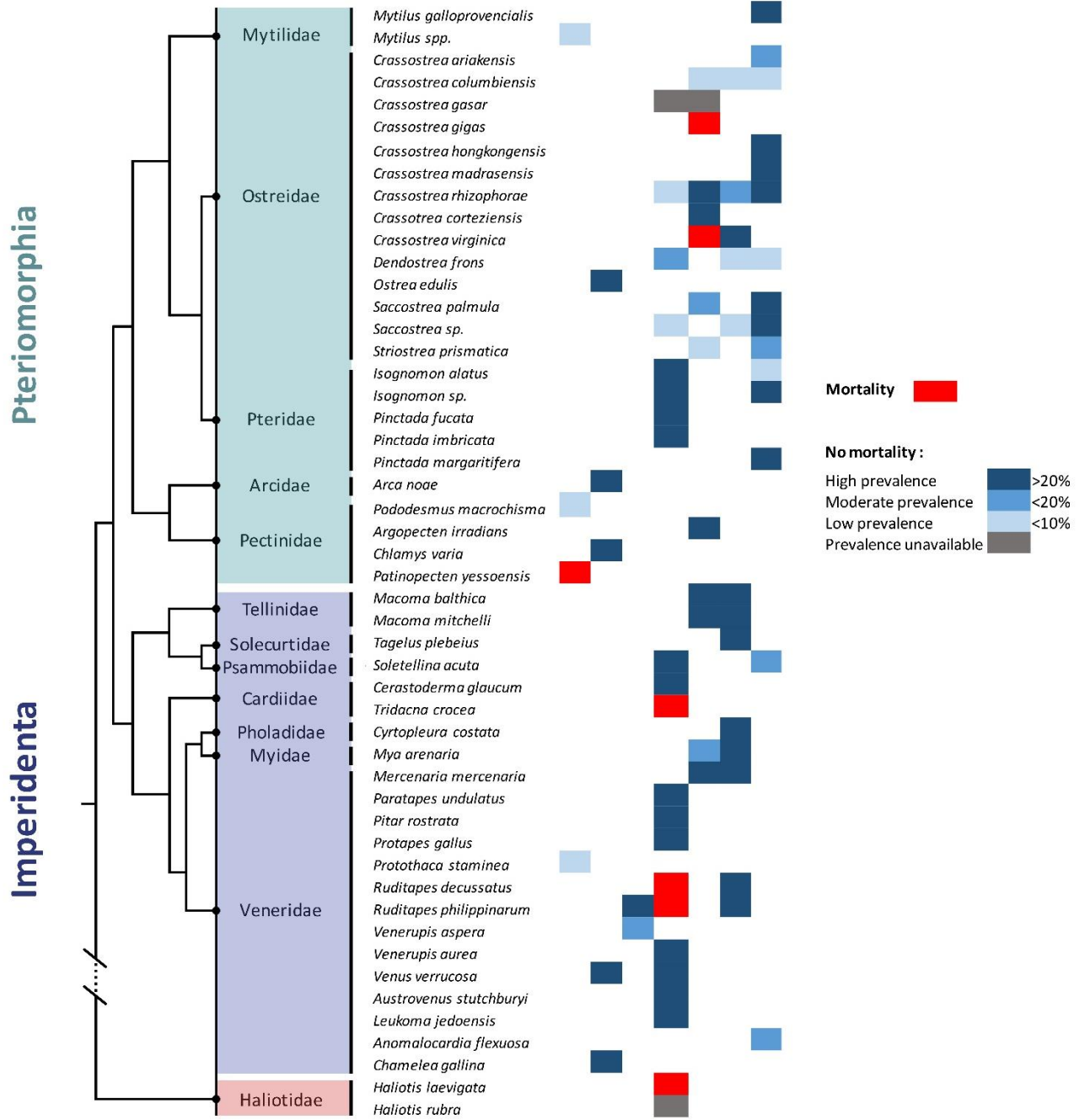
In 1946, following a mass mortality event (MME) of oyster stocks in Louisiana (Gulf of Mexico, U.S.A.), Mackin *et al.* (1950) identified a protist present in host tissue sample as the causative agent. First affiliated to a fungi lineage and named *Dermocystidium marinum* (Mackin *et al.*, 1950), electronic microscopy observations then showed morphological characteristics such as the presence of a subpellicular membrane, micropores and a conoid-like structure (zoospore stage) suggesting a re-affiliation of this protist into the Apicomplexa phylum (Alveolata) (Perkins and Menzel, 1967; Perkins, 1976). Although doubts remained about the accuracy of this affiliation (Siddall *et al.*, 1997), molecular phylogenies based on ribosomal and actin sequences revealed that *Perkinsus* species were more closely related to dinoflagellates than apicomplexans (Reece *et al.*, 1997). More recently, multiple protein phylogeny indicated that *Perkinsus* is an early branch in dinoflagellate lineage (Figure 2) (Saldarriaga *et al.*, 2003). Today, seven species are described within the genus *Perkinsus*: *P. marinus*, *P. olseni*, *P. qugwadi*, *P. chesapeaki*, *P. mediterraneus*, *P. honshuensis* and *P. beihaiensis* (see Figure 1B and 2B for the distribution of these parasites). However, only *P. marinus* and *P. olseni*, the aetiological agents of "Dermo" disease and Perkinsosis respectively, have significant negative impacts on mollusc populations worldwide (Siddall *et al.* 1997). These two infectious agents are today within the list of notifiable diseases in the World Organization for Animal Health (O.I.E.) (O.I.E. 2019a). *Perkinsus* species have generally a BHR among mollusc species, for example *P. olseni* infects various clams (e.g. *R. decussatus*, *R. philippinarum*, *Austrovenus stutchburyi*, *Tridacna maxima*, *T. crocea*, *Pitar rostrata*), oysters (*Crassostrea rhizophorae*, *Saccostrea sp.*), pearl oyster (e.g. *Pinctada imbricata*, *P. funcata*) and abalone (e.g. *Haliotis rubra*, *H. laevigata*) (Cremonte *et al.* 2005, Dungan *et al.* 2007, Lester & Davis 1981, Pagenkopp Lohan *et al.* 2016, Ramilo *et al.* 2015, Sanil *et al.* 2010, Sheppard & Phillips 2008) (Figure 3).

Chapitre 1.1 : Une nouvelle ère des micro-parasites émergents : les cas des Perkinsozoa ?

A.



B.



◀ **Figure 3.** Host range and prevalences of *Perkinsus* species among molluscs. **A)** Schematic phylogeny of Bivalvia (Mollusca) based on Combosch *et al.* in 2017. **B)** Host range matrix according to different levels of prevalence and mortality events for the seven *Perkinsus* species. Hosts infection reference were confirmed in the literature by molecular (qPCR or PCR) and microscopical methodologies (histology or RFTM incubation or cultures) or solely by molecular methods if the genetic signature were extracted from host tissue and sequenced.

Up to date, most of host species described are of commercial interest but others bivalve and gastropod species may also be susceptible to infection, especially in the described geographical range. Although the host range of these parasitic species is extremely large, many questions relative to specificity of infection remain unsolved. Despite their BHR character, host susceptibility needs to be considered to prevent and protect most sensible bivalve stocks. For example, the two genetically related oysters *C. gigas* and *C. virginica* respond with different susceptibility when challenged by *P. marinus*. Although infection is established in both hosts, only infection in *C. virginica* is lethal (Barber and Mann, 1994). Among *Perkinsus* BHR, different levels of prevalence depending of hosts species are recorded across the world (Figure 4).

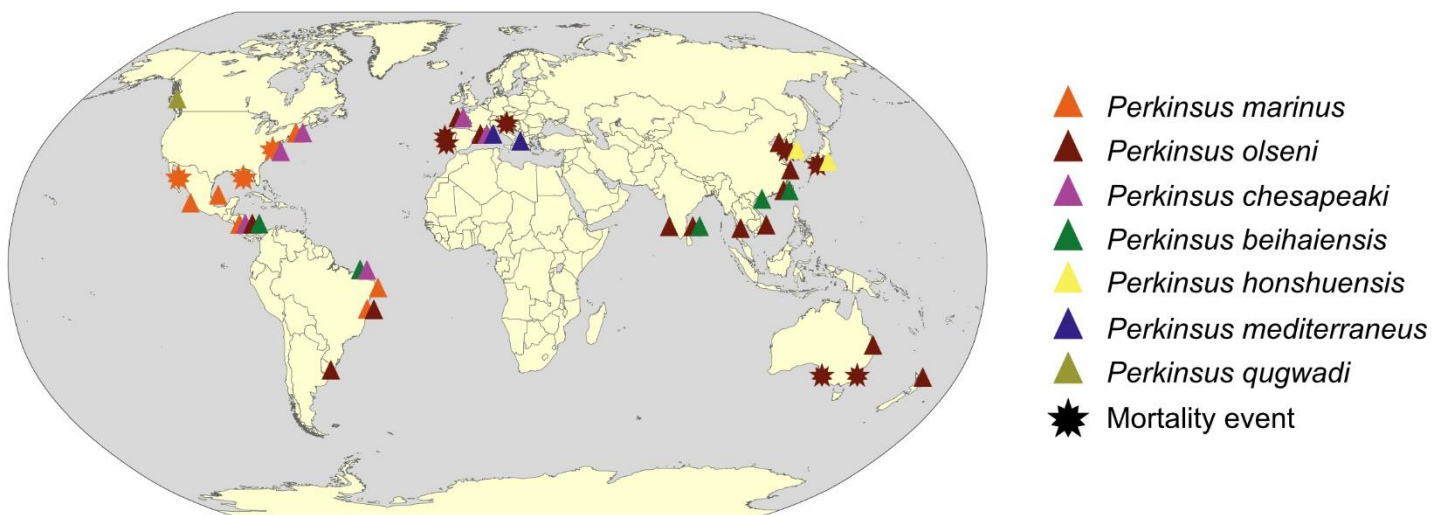


Figure 4. Geographical distribution of the Perkinsidae parasitic protists based on scientific literature from 1950 to 2020. Detection provenance of Perkinsidae (details of references selected in Supplementary Table S1) is indicated by a coloured triangle when the parasite is detected simultaneously by molecular (qPCR or PCR) and microscopical methodologies (histology or RFTM incubation or cultures), and by a star when presence of the parasitic protists is linked to a mortality event. The colour of triangles or stars designates the parasite species.

In the Chesapeake Bay, *P. marinus* and *P. chesapeaki* are infective for different species of oysters and clams living in sympatry. Among these hosts, *P. marinus* rarely infects clams compared to *P. chesapeaki* and exhibits oyster preference (Reece *et al.*, 2008). Species-dependent prevalences are observed in *Perkinsus* spp. which testify host preferences and difference in host susceptibility. Similarly Pecher *et al.* (2008) observed a preferential infection of *C. virginica* supported by a higher prevalence than in *M. mercenaria*. Detected in two different species of Veneridae: *R. philippinarum* and *Venerupis aspera* (Dungan and Reece, 2006; Takahashi *et al.*, 2009; Kang *et al.*, 2016), *P. honshuensis* is the only one to be classified as an intermediate generalist (Figure 1 & 3B). Variability in pathogenicity across the BHR of *Perkinsus* spp. raises the question about the existence of specific strains. For example, *Marteilia refringens* (Paramyxan), responsible of *Marteiliosis*, exhibits a potential profile of BHR parasite because this micro-eukaryotic parasite infects oysters as well as mussels. Nonetheless, two different strains have been observed: the “O” strain mainly found in oysters, with a mortality rate of 90% in *Ostrea edulis*, and the “M” strain mainly found in mussels, with a mortality of 20 to 30% in *Mytilus galloprovincialis* (Guo and Ford, 2016). No such specific strain was detected for *Perkinsus* spp. and the origin of their BHR remains at least partially enigmatic. Globally, *in situ* studies of *Perkinsus* BHR demonstrated clear occurrence for host preference that is not explained by intraspecific variability. *P. marinus* is a perfect example of generalist parasite with preference for oysters but able to infect susceptible hosts more distant phylogenetically (Reece *et al.*, 2008). However, in view of the increase in epizootic diseases worldwide, the consideration of the less preferred hosts is absolutely essential for two reasons: 1) they may be the starting point of a host shift and 2) they represent latent reservoirs for these pathogens.

Perkinsus infection appears to occur directly by filtration without an intermediate host with gills and labial palps playing a crucial role as entrance portal for the parasite (Chintala *et al.*, 2002; Wang *et al.*, 2018). Four different life stages are today described occurring inside and/or outside the host with an exception for *P. qugwadi* where all stages can be observed in host tissues (Blackbourn *et al.*, 1998). All of them are able to induce new infections into a healthy host. The trophozoite life stage, a vacuolated spherical cell having a "signet ring" appearance, proliferates by vegetative multiplication (palintomy) in the host tissue. The cell undergoes successive partitioning to yield up to 32 daughter cells forming a multinucleated structure named "rosette". Disruption of the cell wall allows the release of immature spherical trophozoites that will gradually enlarge becoming mature vegetative cells (Perkins, 1996). In

case of heavy infection, trophozoites can invade totally host tissues, with occasionally production of cutaneous white nodules (inflammatory reaction), and induce a global decrease in host fitness (e.g. lower filtration activity, retard in growth and reproduction) (Dittman *et al.*, 2001; Lee *et al.*, 2001; Choi and Park, 2010). When the host becomes moribund, the parasite enlarges and develops a thick cell wall becoming hypnospore life stage (Valiulis and Mackin, 1969; Perkins, 1996). This step is rarely observed in the host tissues samples even if some environmental factors (e.g. anaerobic condition, acidic pH, medium nutrient rich) are identified in hypnospore formation (Casas and La Peyre, 2013). However, hypnospore life stage is mostly observed *in vitro* following the incubation of parasitic culture and/or infected tissue samples in the Ray's fluid thioglycollate medium (RFTM) that mimics anaerobic conditions (Ray, 1952). When hypnospores are transferred into fresh seawater under favorable conditions of temperature and salinity (Auzoux-Bordenave *et al.*, 1996), they undergo multiple divisions allowing the formation of free-living bi-flagellate life stages, the zoospores, into a cellular structure the zoosporangium. At maturity, zoosporangium releases zoospores in the medium allowing infection of new hosts (Figure 5).

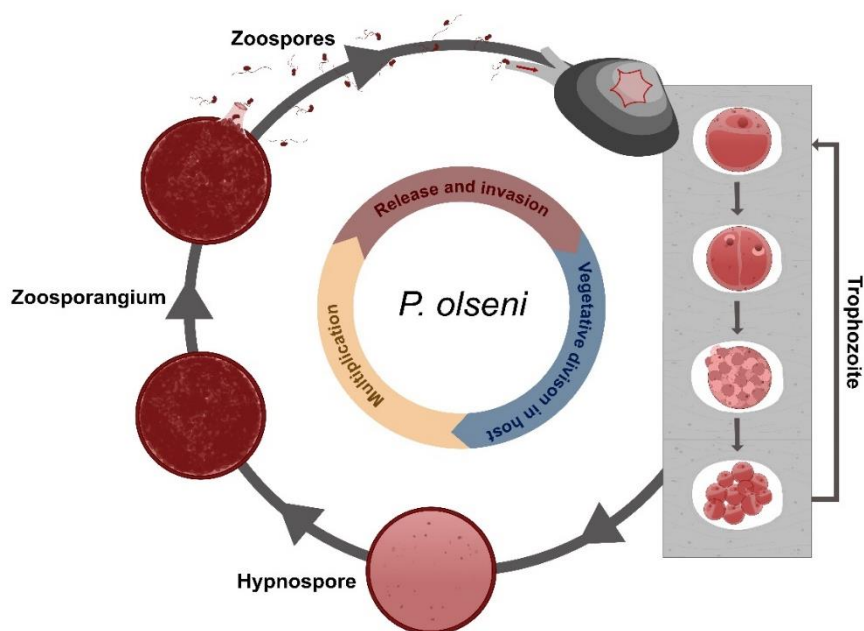


Figure 5. Cartoon illustration of the described life cycle of *Perkinsus olseni* infecting its clam host (adpated from Auzoux-Bordenave *et al.* 1995)

Perkinsus species are one of the most famous examples of successful invasive pest in marine environments. Three different scenario are described through three different *Perkinsus* species: **(1) Expansion:** *P. marinus* has shown a significant increase of its geographic area in the U.S.A along the East coast by more than 500 kilometers in two years (1990-1992). This expansion,

which is now permanent, has been correlated with an increase in winter surface temperature due to milder winters (Ford and Smolowitz, 2007); **(2) Introduction:** *P. chesapeaki* appears to have been accidentally introduced in Europe via its vectors *Mya arenaria*, or the hard clam, *Mercenaria mercenaria* from the U.S.A (Arzul *et al.*, 2012). The sporadic detection on *P. chesapeaki* does not reflect an expansion dynamic. To date, no mortality event affiliated to *P. chesapeaki* has been recorded; and **(3) Introduction and expansion:** *P. olseni* has been co-introduced in Europe with its host the Manila clam, *Ruditapes philippinarum*, in 1972 for the development of aquaculture (Ruano *et al.*, 2015). After its first detection in the late 1980s, the first mortalities of clam stocks were attributed to this parasite at Rio de Faro in Portugal (Ruano and Cachola, 1986). Since then, *P. olseni* has produced mortalities in Spain, Portugal and Italy, infecting both the exotic Manila clam, *Ruditapes philippinarum*, and the native European clam, *Ruditapes decussatus* (Azevedo, 1989; Figueras *et al.*, 1992; Preto *et al.*, 2014). Following these first mortalities, *P. olseni* was detected along the French Atlantic coast with infection prevalence up to 100% (Lassalle *et al.*, 2007), in contrast to the non-detection of *Perkinsus* spp. in most of (these) previously studied sites (Goggin, 1992). Similarly, histological studies conducted by Vilela (1951) on *Haplosporidium* infection in the Formosa lagoon in Portugal, where the prevalence of *P. olseni* infection is very high, ranging from 60% to 90% depending of the season (Leite *et al.*, 2004), did not mention any infection with *Perkinsus* spp.. Finally, in 2011, using microsatellite genetic diversity analysis, Vilas *et al.* described low genetic diversity of the *P. olseni* parasite in Spain and Portugal compared to samples collected in Japan and New-Zealand which might result of a founder effect testifying an introduction event of this parasite (Vilas *et al.*, 2011). These examples of *Perkinsus* spp. dissemination demonstrate the width of their powerful invasive capacity.

The ubiquitous nature (exploitation of important range of abiotic and biotic niches) of *Perkinsus* species is a threat for shellfish farming economy and for ecosystem equilibrium. Most *Perkinsus* spp. (e.g. *P. marinus*, *P. chesapeaki*, *P. qugwadi*, *P. mediterraneus* and *P. beihaiensis*) are able to infect bivalves across the sub-orders of Pteriomorphia and Imparidentia (Bivalvia). Among all of them, *P. olseni* is the well-known “true” generalist because of its high prevalence (>20%) in different hosts belonging to Pteriomorphia, Imparidentia and Haliotidae family (Gasteropoda). Conversely, *P. honshuensis* was solely detected in two related species belonging to Imparidentia sub-order (Figure 3). The phylogenetic distribution of hosts infected demonstrates the relevance of the evolutive history of molluscs at macro- and micro-scale (plesiomorphy, homoplasy, co-evolution) in the *Perkinsus* BHR. Bivalves are known to provide

important ecosystem services through their status as habitat engineers, as filter feeders and as a connecting compartment between primary producers and predators (Lawton and Shachak, 1994; Lowery *et al.*, 2007; Gallardi, 2014). By cascade effect, parasitic infection can have deleterious effects on the different functions attributed to these populations: **1**) by altering their bioturbatory activity and thus by modifying biogeochemical cycles at the water-sediment interface which will have consequences on climate regulation (Dairain *et al.*, 2019), **2**) by impacting their filtration activity participating to the nitrogen cycle (= production of NH_4^+ leading to an increase in primary production) and helping to reduce turbidity (= increase of light penetration) (Gallardi, 2014) and **3**) by altering their physiological traits (e.g. growth, reproduction, survival) which will modify the structure of habitat, biotic interaction and the species richness associated (e.g. Thomas *et al.* 1998). Additionally, to this infectious threat, benthic biodiversity is threatened by human activities. Indeed, anthropogenic eutrophication and temperature increase are all factors that can weaken benthic organisms' immune system and lead to a decrease in their resistance to parasitic protist as *Perkinsus* spp. (Morley, 2010). Furthermore, the host range of these parasitic organisms might be wider than described in the scientific literature with the existence of other “non-commercial” potential hosts belonging to sub-orders infected by *Perkinsus* spp. Thus, even though many arguments allow classify *Perkinsus* species as BHR parasite, we can't determine the real consequences on stocks of “non-optimal” hosts especially when it is related to sporadic infections with low prevalence. Due to research focus on economic valuable species of molluscs like *C. virginica* or *R. philippinarum*, consequences of these infections on the global host range of *Perkinsus* spp. are still mainly undiscovered. However, these non-commercial molluscan species also play a functional role in benthic ecosystems and might be key component, as vector, in the spread and success in the disease outcome.

3. Parviluciferaceae, the group of microalgae parasites

In 1999, Norén *et al.* identified small round structures abnormally present within the toxic dinoflagellate *Dinophysis* spp. on the west coast of Sweden (Norén *et al.*, 1999). Molecular and microscopic analyses revealed that this parasitic organism were affiliated to Perkinsids group and erected a new phylum, the Perkinsozoa (syn. Perkinsids, Perkinsea) (Norén *et al.*, 1999). So far, seven species belonging to the *Parviluciferaceae* family, including four genera, have been described and cultivated (Jeon *et al.*, 2018). The first genus described was *Parvilucifera* genus encompassing *P. infectans* (Norén *et al.*, 1999), *P. multicavata* (Jeon and Park, 2020), *P.*

sinerae (Figuroa *et al.*, 2008), *P. rostrata* (Lepelletier *et al.*, 2014b) and *P. corolla* (Reñé *et al.*, 2017a). Species belonging to this genus are genetically very close (> 97% identity based on their SSU rDNA sequences) (Jeon *et al.*, 2018). Recently, three new genera were described, the genus *Snorkelia* containing *P. prorocentri* (Leander and Hoppenrath, 2008) renamed *Snorkelia prorocentri* (Reñé *et al.*, 2017b), the genus *Dinovorax* including *D. pyriformis* (Reñé *et al.*, 2017b) and finally *Tuberlatum* encompassing *T. coatsi* (Jeon and Park, 2019). These three late genera seem to have a basal position in the *Parviluciferaceae* (Jeon *et al.*, 2018) (Figure 2B). However, this list of parasitic species might not be exhaustive and therefore numerous species are waiting to be described.

Members of the *Parviluciferaceae* family share many common traits including development and characteristic of their life cycle, and their infection strategy (Reñé *et al.*, 2017b). The infection begins with the entry of the zoospore into the dinoflagellate host cell (Figure 6). Once inside, the parasite develops in the cytoplasm forming a spherical cell, the trophont (=trophocyte), which consumes its host for its own growth. At the end of the feeding stage, a free-living non-motile cell, the sporocyte (=immature sporangium), occupying the whole intracellular space, is released by breaking the host's theca. Next, the cell undergoes morphological transformations into a spherical multinucleated dark structure called the sporangium (=mature sporocyte). The sporangium remains in dormancy until the detection of host chemical signal (Garcés *et al.*, 2013b). After a short maturation (~ 48h), sporangium with open aperture(s) releases several hundred new infectious cells (zoospores) (Alacid *et al.*, 2017).

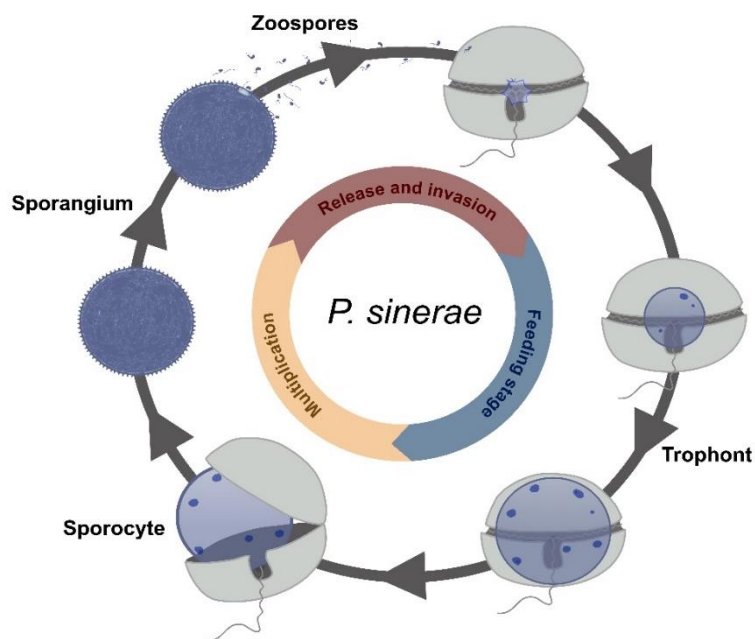


Figure 6. Cartoon illustration of the described life cycle of *Parvilucifera sinerae* infecting its dinoflagellate host (adapted from and Alacid *et al.* 2015)

To date, all described species are parasites of dinoflagellates including toxic species and possess a BHR (here belonging to “true” generalist class) (Figueroa *et al.*, 2008; Garcés *et al.*, 2013b; Jeon *et al.*, 2018; Rodríguez and Figueroa, 2020) except *S. prorocentri* which was known to only infect the marine benthic dinoflagellate *Prorocentrum fukuyoi* (Leander and Hoppenrath, 2008) (Figure 1B). Members of the *Snorkelia* genus could also be BHR because it infects *Prorocentrum micans*, *Dinophysis sacculus* and probably *Levanderina fissa* (Reñé *et al.*, 2017b). For the “true” generalists *P. sinerae*, *P. corolla* and *P. rostrata*, the host range was determined mainly by *in vitro* cross-infection. *In vitro* experiments could result in artificial linkage between a host species and a parasite which is not representative of the ecological reality (Råberg *et al.*, 2014; Alacid *et al.*, 2016). Infection of non-preferred hosts could be based on chemical and physiological processes shared by several related hosts (e.g Garcés *et al.* 2013a). Furthermore, when a parasite infects a host but is not able to product viable parasitic cells, such “host” cannot be considered as a “true” host for the parasite; e.g. infection of a chlorophyte strain of *Pyramimonas* by *P. corolla* (Rodríguez and Figueroa, 2020). The infection process is probably the result of a plesiomorphic mechanisms among *P. corolla*'s hosts but something is missing for the achievement of its whole life cycle. Furthermore, despite of their BHR established mostly in laboratory experiments, the situation. In this review we considered *Parvilucifera* species as generalists when they realise a viable life cycle in large repertoire of host species. A BHR gives them the full potential of host-shifting in new environments although there are some evidences that the situation is not so clear-cut with some host preference in nature (Alacid *et al.*, 2016). However, much remains to be done to evaluate the contribution of the parasite's plesiomorphic and convergent traits to the success of generalists.

Despite a “hot-spot” of detection in Europe, the distribution of these parasites of dinoflagellates is worldwide in marine environments (Figure 7). As major contributors, with diatoms, to the fixation of inorganic carbon through photosynthesis (Falkowski, 1994), dinoflagellates are one of the most important components of marine phytoplankton as primary producers and grazers, representing the base of food webs. *Parvilucifera* parasites could participate in regulating dinoflagellate populations in blooming period, as shown in Spain where 5 to 18% of the noxious dinoflagellates species, *Alexandrium minutum*, were killed by *Parvilucifera* sp. during natural bloom (Alacid *et al.*, 2017). For methodological limitation reasons, most studies describing *Parvilucifera* spp. have been carried out when prevalence is the highest on blooming host species, thus increasing the chances to detect and describe new parasitic species. Unfortunately, this bias hides a possibly important part of hosts and parasites diversity. Indeed, in some studies,

the host range of these parasites appears to be much wider, including non-harmful species (e.g. Figueroa *et al.* 2008; Garcés *et al.* 2013b; Rodríguez & Figueroa 2020), even if the *in vitro* specificity tests do not take into account the complexity of planktonic interaction network and the *in situ* parasitic host-preferences. This lack of knowledge on *Parvilucifera* distribution, diversity and trophic niche might become problematic, given its BHR nature and the global trades. Indeed, ballast waters, which are one of the most important vector of introduction in marine systems (Carlton, 1985; Barry *et al.*, 2008), could contribute to the dispersal of *Parvilucifera* trophonts or dormant sporangia (e.g. Darling *et al.* 2018, Lohan *et al.* 2016), thus increasing the risk of settlement in new areas. Given the importance of dinoflagellate species in the primary production and productivity of marine ecosystems, new introductions of these BHR parasites could take part to the deregulation of the planktonic compartment and induce significant shifts in food webs.

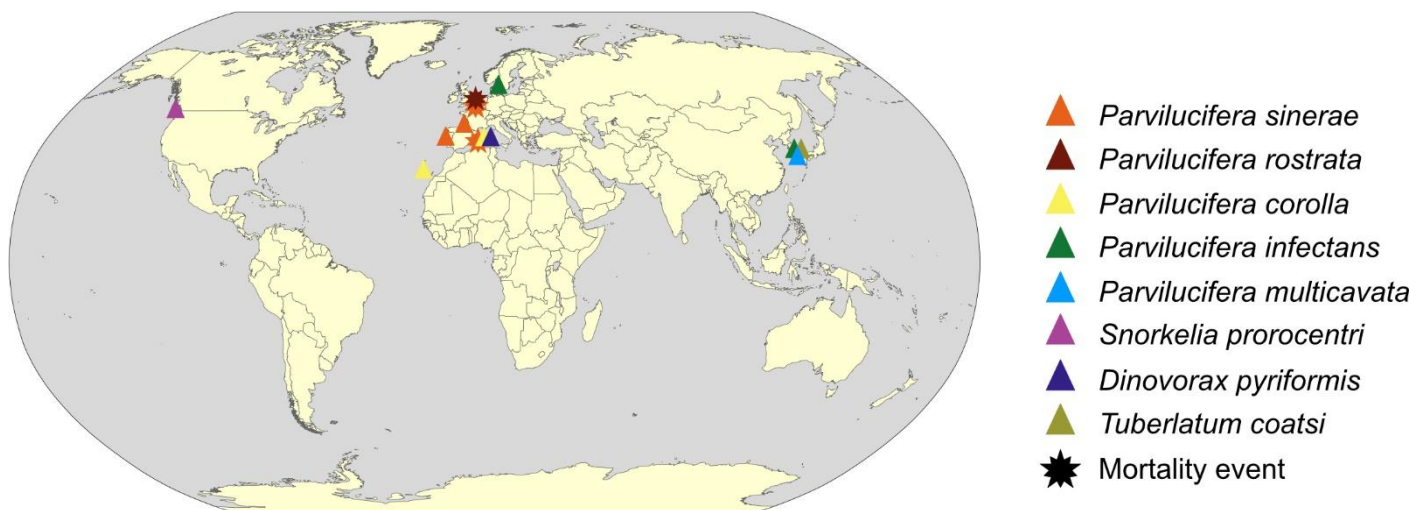


Figure 7. Geographical distribution of the Parviluciferaceae parasitic protists based on scientific literature from 1950 to 2020. Detection provenance of Parviluciferaceae (details of references selected in Supplementary Table S1) is indicated by a coloured triangle when the parasite is detected simultaneously by molecular (qPCR or PCR) and microscopical methodologies (histology or RFTM incubation or cultures), and by a star when presence of the parasitic protists is linked to a mortality event. The colour of triangles or stars designates the parasite species.

4. *Xcellia*, *Gadixcellia*, newly described fish parasites

In 1969, Brooke *et al.* observed pseudotumour-like formations (xenomas) on the epidermal surface of two flatfish species, the black-tipped flounder, *Psettichthys melanostictus*, and the Pacific plaice, *Platichthys stellatus* (Brooks *et al.* 1969). Thirty-five years later, a protozoan named X-cell was identified as responsible for this pathology inducing also multiple lesions and swelling of the gill filaments (Miwa *et al.*, 2004). In 2017, using small and large concatenated subunits (SSU and LSU) rDNA phylogeny reconstruction, Freeman *et al.* investigated the phylogenetic position of this enigmatic X-cell protist and showed that it formed two highly distinct clades, one corresponding to the pseudobranchial parasites of Gadiformes, and the other to gill and epidermal X-cells from Perciformes and Pleuronectiformes called *Gadixcellia* and *Xcellia* respectively. Despite an unusually high genetic divergence between the two genera, with similarity of 74,9% for the SSU rDNA sequences, these two closely sister clades form a new family called Xcellidae branching in a basal position within the Perkinsozoa lineage (Freeman *et al.*, 2017b) (Figure 2B).

Parasites belonging to these two genera have been detected in more than 20 fish species belonging to five orders of teleost: Pleuronectiformes (flatfishes) (Freeman, 2009; Freeman *et al.*, 2011), Perciformes (perch-like fish) (Katsura *et al.*, 1984), Gadiformes (cods) (Freeman *et al.*, 2011), Siluriformes (catfishes) (Diamant *et al.*, 1994) and Salmoniformes (salmonids) (Dyková *et al.*, 1993). Up to date, four species have been described. (Figure 2). The parasite *Xcellia pleuronecti* infection has been confirmed in *Hippoglossoides dubius* and *Pseudopleuronectes obscurus* but also suspected in many other species from the same area: *Cleisthenes herzensteini*, *Cleisthenes pinetorum*, *Glyptocephalus stelleri*, *Kareius bicoloratus*, *Hippoglossoides elassodon*, *Liopsetta pinnifasciata*, *Platichthys stellatus*, *Parophrys vetulus*, *Pseudopleuronectes schrenki* and *Verasper moseri*. Equally, *Xcellia lamelliphila* infection is detected in *Limanda limanda*, *Lycodes* spp., *Macruronus novaezelandiae*, *Merluccius gayi gayi* and *Trematomus* spp. (Freeman *et al.*, 2017). These two parasites are BHR parasites and classified as “true” generalists (Figure 1B). Conversely, *Xcellia gobii* and *Gadixcellia gadi* infecting respectively *Acanthogobius flavimanus* and *Gadus morhua* are NHR parasite and classified as specialist (Freeman *et al.*, 2017) (Figure 1B). This classification will certainly evolve as new knowledge on the *Xcellia* and *Gadixcellia* clades become available.

Although the life cycle of this parasite remains still unknown, Freeman *et al.* (2017) suggested that infection occurs via contact between fish and the benthos. Indeed, X-cell infection occurs in fish species with at least one benthic stage during their life cycle (Fahay, 1983; Lough *et al.*, 1989). An experimental infection *in situ* in tanks revealed that only fish exposed, even transiently, to the benthos, were infected and showed symptoms of disease (Eydal *et al.*, 2010). The infected fish samples are mainly young and adult individuals, indeed the first pathogenic symptoms (pseudobranchial xenomas) appear macroscopically in young wild cod (around 6 months old, 6.5 to 13.0 cm length) from Icelandic waters with a prevalence peak of 23% at the age of 22 months whereas older or larger fish (40 to 76 cm length) have a lower prevalence around 7% and 1% respectively (Eydal *et al.*, 2010). Similar trends had been observed in Atlantic cod (Morrison *et al.* 1982) and the Pacific cod (Stich *et al.*, 1976). However, the question remains open whether the mortality induced by the X-cell infectious agent on juvenile could remove an age-class from the field-sampling and bias the observed prevalence. Although mortality induced by these infectious agents still need to be demonstrated *in situ*, the transfer of wild infected individuals of *Gadus morhua* in tank has shown an important mortality (Eydal *et al.* 2010).

In addition, *Xcellia* and *Gadixcellia* protists are detected in a restricted geographical area (Northern Europe and Japan) (Figure 8) but the monitoring of these X-cell infections is hard to carry out and stay largely incomplete because of migratory fish hosts in the worldwide ocean. Their host range is mainly unknown but we can hypothesize that it may be broader than that currently identified. An extended host range would not be surprising considering the Perkinsozoa phylum constituted of a majority of BHR parasites closely related phylogenetically (e.g. the Perkinsidae) (Figure 2B). Overall, the consequences of the X-cell infection on fish populations are not well known. However, as it affects important halieutic resources, it would be necessary to monitor more efficiently its occurrences. These parasites could infect vulnerable species such as Atlantic cod fish *G. morhua*, extensively farmed, but mainly in north of Europe (see http://www.fao.org/fishery/culturedspecies/Gadus_morhua/en). Commercially important fish concerned by the global trades could act as reservoir allowing the dissemination in farmed and wild fish populations.

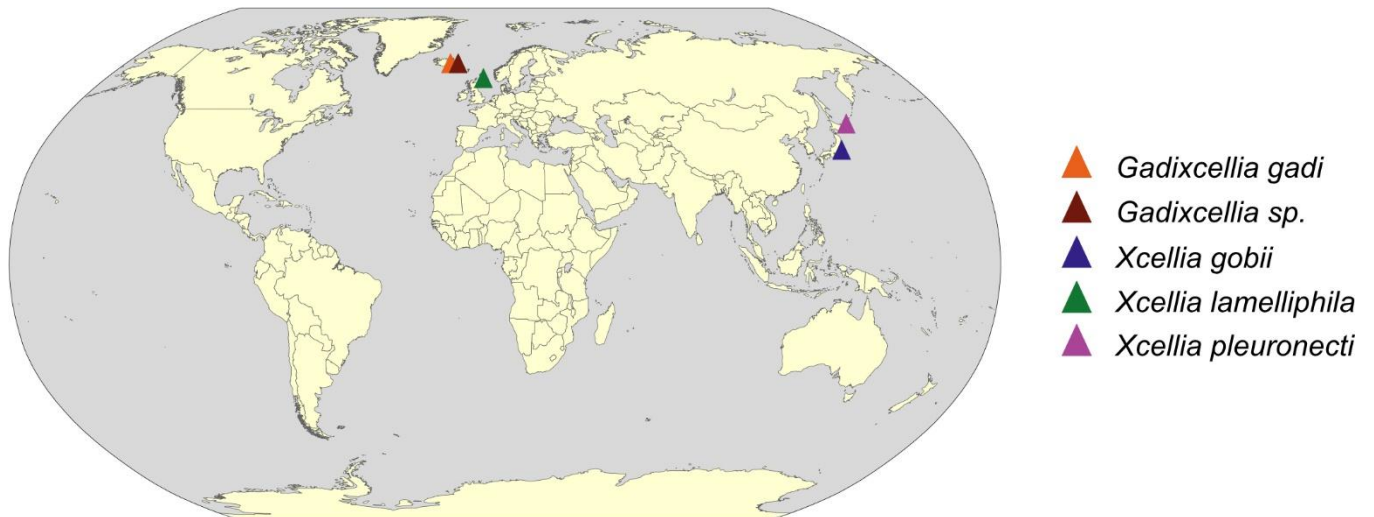


Figure 8. Geographical distribution of the Xcellidae parasitic protists based on scientific literature from 1950 to 2020. Detection provenance of Xcellidae (details of references selected in Supplementary Table S1) is indicated by a coloured triangle when the parasite is detected simultaneously by molecular (qPCR or PCR) and microscopy methodologies (histology or RFTM incubation or cultures), and by a star when presence of the parasitic protists is linked to a mortality event. The colour of triangles or stars designates the parasite species.

5. Severe Perkinsea Infection (SPI), an infectious agent of tadpoles' populations

With up to 50% of all threatened species, amphibian populations are emblematic representatives of the sixth mass extinction event to which highly virulent wildlife diseases contribute (Stuart *et al.*, 2004; Gewin, 2008; Chambouvet *et al.*, 2020). Recent works have mainly identified two pathogens as drivers of this decline, the fungus *Batrachochytrium dendrobatidis* and the Ranavirus (e.g. Bosch *et al.* 2001; Fisher *et al.* 2009; Gray *et al.* 2009; Olson *et al.* 2013).

However, in April 2006, in a pond in northeast Georgia (U.S.A.), Davis *et al.* observed a massive mortality event of southern leopard tadpoles, *Lithobates sphenoccephalus* (anciently *Rana sphenoccephala*), attributed to an unknown protist affiliated by phylogenetic analysis to the Perkinsozoa lineage (Davis *et al.*, 2007). Using phylogenetic analysis, it has been shown that this organism belongs to a larger clade called Pathogenic Perkinsea Clade (PPC) (Isidoro-Ayza *et al.*, 2017) or Novel Alveolate Group 01 (NAG01) (Chambouvet *et al.*, 2015) obtained

from disparate freshwater environments or internal organs (i.e. liver tissues) of a wide variety of Neobatrachia suborder (Chambouvet *et al.*, 2015; Isidoro-Ayza *et al.*, 2017). Conversely to the “*Rana sphenocephala* pathogen”, the NAG01 is divided into three distinct phylogenetic clades (A, B and C) considered as “true” generalists and BHR parasites (Figure 1B). Representatives of one subclade of this large group have been identified as responsible for the die-offs of tadpole life stage throughout the U.S.A. (Isidoro-Ayza *et al.*, 2017). Infected tadpoles showed lethargic swimming with enlarge and histopathologic lesions of the liver, mesonephros, spleen, pancreas, gills, gastrointestinal tract, skeletal muscle, dermis and peritoneum (Davis *et al.* 2007, Green *et al.* 2002, Isidoro-Ayza *et al.* 2017, 2019 ; Jones *et al.* 2012). These pathological symptoms, named "Severe Perkinsea Infection" (SPI), have been reported from summer to early autumn in boreal and temperate regions, and from late winter to early spring in subtropical areas (Isidoro-Ayza *et al.*, 2017) (Figure 9). Although this parasite is mainly described as infecting tadpole life stage, one report highlights infection of the adult populations with granulomatous lesion in legs (Jones *et al.*, 2012). These results suggest that either most of the tadpoles dies before the metamorphosis or that mature immune systems acquired after the metamorphosis may drive back the infection (Isidoro-Ayza *et al.*, 2017). Although the relationship between the infectious agent and the disease is not yet well established, symptoms of the disease could result from co-infection between the Perkinsozoa parasite and others infectious agents, such as the FV3-like virus, responsible for frog population declines, which infects both larval and adult amphibians (Gray *et al.*, 2009; Lesbarrères *et al.*, 2012), and/or alteration of tadpoles immune systems (Isidoro-Ayza *et al.*, 2017).

During monitored SPI outbreaks, mortality rate can reach up to 95% of the population leading to the loss of an entire age class or, in case of chronicle infection, to a reduced recruitment (Green *et al.*, 2002; Isidoro-Ayza *et al.*, 2017, 2019). Now recognized as the third most common infectious disease of anuran species, SPI infectious agent is described as an emerging pathogen. It is therefore now fundamental to understand the relationship between this infectious agent and the outcome of the disease and how others pathogen communities and/or environmental factors could affect disease susceptibility. Furthermore, as the globalization of trade where amphibian species, generally involved in meat or pet trade, could spread the parasitic invaders via reservoir species into native and/or naive amphibian populations, the study of this pathogen is crucial.

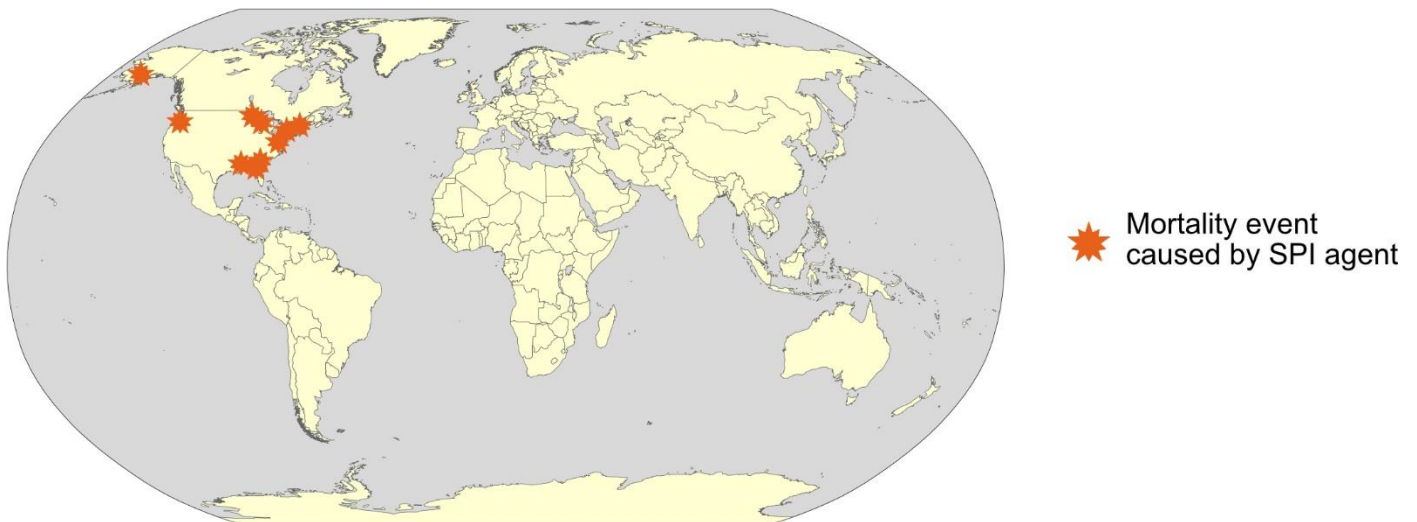


Figure 9. Geographical distribution of the SPI agent parasitic protists based on scientific literature from 1950 to 2020. Detection provenance of SPI agent (details of references selected in Supplementary Table S1) is indicated by a coloured triangle when the parasite is detected simultaneously by molecular (qPCR or PCR) and microscopy methodologies (histology or RFTM incubation or cultures), and by a star when presence of the parasitic protists is linked to a mortality event. The colour of triangles or stars designates the parasite species.

6. Unveiled environmental diversity: the next reservoir of pathogens?

Molecular methodologies have recently highlighted that our vision of Perkinsozoa diversity based on culturable methods is still only the tip of the iceberg. In both marine and freshwater environments, analysis of genetic diversity based on SSU rDNA sequences of the smallest sizes of plankton (<5 μm), revealed a previously unknown diversity of these organisms (Moon-van der Staay *et al.*, 2001; Lefranc *et al.*, 2005; Lepère *et al.*, 2010).

In freshwater ecosystems, Perkinsozoa sequences appear to be highly diverse and abundant, suggesting an essential role in the trophic food web (e.g. Jobard *et al.* 2020; Lepère *et al.* 2008, 2010; Mangot *et al.* 2011). Recent studies have highlighted that freshwater clades could be a parasitic protist of the colonial green algae *Sphaerocystis* sp. (Jobard *et al.*, 2020). These results are consistent with the description of *Rastrimonas* gen. nov. (Brugerolle, 2003), previously described as *Cryptophagus subtilis*, infecting the free-living Cryptophyte *Chilomonas paramecium* (Brugerolle, 2002). However, apart from these two letter descriptive

publications, no molecular analysis has yet been carried out to definitively affiliate *Rastrimonas* sp. within the Perkinsozoa lineage.

In marine environments, molecular signatures of Perkinsozoa were also found in extreme environments such as hydrothermal vents or anoxic fjords (López-García *et al.*, 2003; Zuendorf *et al.*, 2006) but most of environmental genomics studies target water-column samples (e.g. surface or deep chlorophyll maximum (DCM) depths) (e.g. De Vargas *et al.* 2015, López-García *et al.* 2001, Moon-van der Staay *et al.* 2001). This lack of genetic signatures in marine environmental databases represent a real paradox, since the two main cultivable groups described (e.g. *Perkinsus* spp., *Parvilucifera* spp.) are marine. In 2014, targeting the V4 hypervariable region of the rDNA and rRNA templates using 454 sequencing technology, Chambouvet *et al.* (2014) evaluated the genetic diversity of the eukaryotic microbial community in two sampling depths (surface and DCM) and in the sediment across four different locations across Europe (Oslo [Norway], Naples [Italy], Barcelona [Spain] and Roscoff [France]). The analysis revealed an unexpected genetic diversity of ribosomally active organisms belonging to Perkinsozoa wider than *Parviluciferaceae* and *Perkinsus* clusters mainly detected in the sediment. The ribosomal RNA sequences present in these clades belong to metabolically active organisms, which certainly play an active role in the ecosystem functioning. This cryptic diversity, only detected by their genetic signatures, raises new scientific questions about the host range of Perkinsozoa and its impact on the aquatic food web.

7. Conclusion

All currently known members of Perkinsozoa are described as parasitic species infecting protists, molluscs and vertebrates. They are detected in all ecosystems from the tropics to high latitudes and from freshwater to marine environments (Mangot *et al.* 2011). Perkinsozoa shares common characteristics which represent serious threats for biodiversity and human activity.

(1) They are predominantly BHR. Perkinsozoa consist of four described clades of BHR generalists. At high taxonomic level (Class: Perkinsozoa, syn. Perkinsea), such homogeneity is rare compared to other major parasitic clades in ecosystems. For example, the *Parviluciferaceae* contribute, as well as other zoosporic parasites like *Amoebophrya* spp. or *Dinomyces* sp., to supply the carbon input in the general food webs and to contribute in the energy transfer (Jephcott *et al.*, 2016). Although all of Perkinsozoa yet described are BHR

generalist parasites (except *Snorkelia prorocentri*), there are strong evidences of host preference (e.g. Alacid *et al.*, 2016) and / or susceptibility (e.g. Calvo *et al.*, 1999) mostly established in the laboratory. In nature, the situation is more complex with evidences of some degree of host specificity. Hence, it is now fundamental to establish the "true" parasitic host range in the environmental field. However, this core parameter could be tremendous to establish because these parasites could be unnoticed if they infect potential "reservoir" host at low prevalence. Contrary to Perkinsozoa, some strains of *Amoebophrya* species (Alveolata), exhibit different specificity to host species leading to a mix of parasitic protist displaying various degrees of host specialization (e.g. Coats and Park, 2002; Chambouvet *et al.*, 2008; Farhat *et al.*, 2018). In the same way, while lot of chytrid fungi (Chytridiomycota) infecting phytoplankton are mainly considered as host specific (Ibelings *et al.*, 2004), *Dinomyces arenysensis* has a BHR focused on dinoflagellates species (Lepelletier *et al.*, 2014). This feature may represent a survival advantage in coastal planktonic ecosystems where environmental conditions are highly variable (Dia *et al.*, 2014; Farhat *et al.*, 2018). Same trend is observed for Perkinsidae which is one of the major pest involved in mollusc diseases with the Haplosporidiidae. Contrary to Perkinsids, the Haplosporidiidae family is composed of specialists and generalists. For example, *Bonamia* species infects several oysters species (Engelsma *et al.*, 2014) whereas *Haplosporidium* species are able to infect distant phylogenetic hosts, including molluscs, crustaceans or even polychaetes (Arzul and Carnegie, 2015). Even if the Haplosporidiidae host repertoire seems really diverse, at the species level, those parasites are closer to NHR parasite contrary to *Perkinsus* species. Even if the natural selection favours NHR specialists, the Perkinsozoa phylum is largely dominated by BHR generalists. This peculiar characteristic is probably strongly related to their common evolutionary history and may represent an ecological advantage that enable survival improvement via a wide variety of "potential" hosts.

(2) They are pathogenic for many keystone, engineer or endangered species. Like other important parasites listed at international or national level (e.g. *Batrachochytrium dendrobatidis* or *Marteilia refringens*), Perkinsozoa contribute to the loss of biodiversity (e.g. the SPI agent) and to the massive loss of fishery and aquaculture resources (e.g. *P. olseni* and *P. marinus*). Recent years have seen the advent of diseases induced by *Batrachochytrium dendrobatidis* and *B. salamandrivorans* (agent of chytridyomycosis), listed in the O.I.E-notifiable disease list, which are mainly responsible for the collapse of amphibian populations (Scheele *et al.*, 2019). In 2020, Chambouvet *et al.* reviewed new infectious diseases concerning Apicomplexans (Coccidians, Gregarines) and Perkinsozoa (SPI agent - NAG01) may worsen

amphibians situation. Today, many species of frogs, like *Lithobates capito* or *L. sevosus*, has been listed in “Near threaten” to “Critically endangered” status in the Red List of the International Union for Conservation of Nature (IUCN)(Chambouvet *et al.*, 2020). Their involvement in biodiversity loss may contribute to major shifts in biological communities.

(3) They are responsible of economical loss. Economic valuable species are affected by Perkinsozoa infection which may lead to terrible collapse of fishery industries. As protozoans caused most of the historically significant diseases in molluscs, this parasitic group is of major concern for the shellfish exploitation (Carnegie *et al.*, 2016). The Dermo disease (aetiological agent : *P. marinus*) is one of the major marine molluscan diseases with MSX (Multinucleated Sphere Unknown caused by *Haplosporidium nelsoni*), Marteiliosis (*Marteilia refringens* and *M. sydneyi*), and Bonamiosis (*Bonamia ostrea* and *B. exitiosa*) (Arzul and Carnegie, 2015; Guo and Ford, 2016). Globally, Haplosporidian and Perkinsidae are two major threats for mollusc health even if the mortality rate produced by *P. marinus* (max. 60%) on oysters is lower than the mortality rate of *Haplosporidium nelsoni* or *Marteilia refringens* (90-100%) on the same resource (Guo and Ford, 2016). Indeed, in the Chesapeake Bay, the oyster harvest decline (~ 80 000t between 1910-1980 to 15 000t in 1986) was attributed to both parasites *P. marinus* and *H. nelsoni* in a context of inadequate management practices (Héral *et al.* 1990). Recurrent declines of clam harvests are recorded as result of massive mortalities caused by *P. olseni* in Korea (Park *et al.* 1999, Choi and Park, 2005), China (Liang *et al.*, 2001) and Japan (Hamaguchi *et al.*, 1998). The Manila clam landings from culture in 1997 was approximately 14.000t, which is only one-fifth of the clam landings in 1990 (Park and Choi, 2001). In Europe (Spain, Portugal and Italy), the parasite destroyed *R. decussatus* (native) and *R. philippinarum* (exotic) populations (Azevedo, 1989; Figueras *et al.*, 1992; Pretto *et al.*, 2014) and Perkinsosis induces more damage in clam stock than other important disease like the BRD (Brokassenwn Ring Disease) producing approximately 20% of mortality over 2 years (Guo and Ford, 2016).

(4) They are invasive and easily translocated. Today many precautions (e.g. quarantine of animals) must be taken around the world to stem the spread of Perkinsozoa, particularly with regard to Perkinsidae. Since the first mortalities, *P. marinus* has been listed as notifiable pathogen by the O.I.E (O.I.E, 2019b) and the European Commission (Directive 2006/088/EC). However, *P. olseni* is solely classified at international level in the O.I.E-list of notifiable diseases (O.I.E, 2019a,b) but is out of concern for the European Commission

(Carnegie *et al.*, 2016). Some European countries are *Perkinsus olseni*-free and its exclusion from the notifiable diseases list from European Commission can lead to relaxed vigilance within the trading network contributing to its spread in European pristine areas (Carnegie *et al.*, 2016). National surveillance efforts are different between European Members States and mortality events are mainly reported by shellfish farmers (Arzul *et al.* 2016; Lupo *et al.*, 2014). However, these networks have already revealed pathogens in new area, like OsHV-1 μ var or *B. ostreae*, or new pathogens implied in shellfish mortalities, like *Mikrocytos* species (Cigarría and Elston, 1997; Garcia *et al.*, 2018). The constant detection of new pathogens in new areas should be alarming considering the wide host range of *P. olseni* and all other *Perkinsus* species.

(5) Most of Perkinsozoa are only described by their genetic signatures. Environmental sequencing revealed a wide diversity of several potential micro-eukaryotic parasites in the water column and sediment (De Vargas *et al.*, 2015). Thanks to molecular methods, a lot of problematical parasitic groups, e.g. the haplosporidians, are under survey (Hartikainen *et al.*, 2014; Bass *et al.*, 2015). The study of environmental diversity is absolutely crucial to identify potential distribution and emergence of parasites. Recently, *Haplosporidium diporae* infecting amphipods was associated to the previously described environmental clade “haplosporidian clade C” (Hartikainen *et al.*, 2014; Winters and Faisal, 2014; Bass *et al.*, 2015). It is now clear that Perkinsozoa lineage is genetically diverse in aquatic environments and may be composed of clades with a harmful potential whose hosts have not yet been identified. This environmental diversity described across different clusters (e.g. Chambouvet *et al.*, 2014) represents a clear potential parasites. However, their host range and impact of the aquatic food webs are still black boxes that the scientific community need to urgently address considering the impact of already described BHR species belonging to this lineage.

In a context of intensification of the global trade, this kind of opportunistic BHR parasitic protists represent a threat to become successful invasive species eventually leading to a new putative emerging disease. It is thereby now important to investigate their full host range, their cryptic diversity and their role in the global interactive food web network. On the other hand, a growing body of evidence emphasizes the importance of the interactive network between a host, the associated microbial communities and the environmental condition in the determination of infection outcome. Indeed, the outdated "one parasite, one disease" paradigm is not sufficient to explain disease and therefore, these parasites and their studies should be

integrated into a larger scheme (Bass *et al.*, 2019). Knowing under what conditions invasion by these infectious agents could be successful and what threats they could pose to native host populations are two more fundamental questions that need to be addressed.

Chapitre 1.1 : References

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Chapitre 1.1 : Supplementary

Supplementary Table 1. Sources of occurrences of Perkinsiidae, Parviluciferaceae, SPI agent and Xcellidae detected both by one molecular methodology (qPCR or PCR) and by one microscopically methodology (histology or RFTM incubation or cultures).

Clade	Species	Continent	Country	Location	Reference
Perkinsiidae	<i>Perkinsus olseni</i>	Europe	Spain, France, Portugal, Italia	Mediterranean Sea, Atlantic Ocean	Azevedo, 1989; Figueras <i>et al.</i> , 1992; Elandalousi <i>et al.</i> , 2009; Arzul <i>et al.</i> , 2012; Pretto <i>et al.</i> , 2014; Ramilo <i>et al.</i> , 2015
Perkinsiidae	<i>Perkinsus olseni</i>	Asia	China, Korea, India, Japan, Vietnam	Yellow Sea, Bohai Sea, East China Sea, Sea of Japan, Bay of Bengal, Lakshadweep Sea, Pacific Ocean	Hamaguchi <i>et al.</i> , 1998; Park <i>et al.</i> , 2006, 2008; Sheppard and Dungan, 2009; Cho and Park, 2010; Samil <i>et al.</i> , 2010; Wu <i>et al.</i> , 2011; Shama <i>et al.</i> , 2018
Perkinsiidae	<i>Perkinsus olseni</i>	South America	Brazil, Uruguay, Panama	Atlantic Ocean, Caribbean Basin	Cremonte <i>et al.</i> , 2005; Sabry <i>et al.</i> , 2009; da Silva <i>et al.</i> , 2014; Pagenkopp Lohan <i>et al.</i> , 2016
Perkinsiidae	<i>Perkinsus olseni</i>	Oceania	Australia, New-Zealand	Indian Ocean, the Coral Sea, Pacific Ocean	Goggin and Lester, 1995; Murrell <i>et al.</i> , 2002; Dungan <i>et al.</i> , 2007
Perkinsiidae	<i>Perkinsus marinus</i>	North America	USA, Mexico	Chesapeake Bay, Gulf of Mexico, Gulf of Maine, Delaware bay, Gulf of California	Marsh <i>et al.</i> , 1995; Reece <i>et al.</i> , 2001; Gullian-Klanian <i>et al.</i> , 2008; Cáceres-Martínez <i>et al.</i> , 2008; Enríquez-Espinoza <i>et al.</i> , 2010; Marquis <i>et al.</i> , 2020
Perkinsiidae	<i>Perkinsus marinus</i>	Central America	Panama	Caribbean Basin	Pagenkopp Lohan <i>et al.</i> , 2016
Perkinsiidae	<i>Perkinsus marinus</i>	South America	Brazil	Atlantic Ocean	da Silva <i>et al.</i> , 2013, 2014
Perkinsiidae	<i>Perkinsus chesapeaki</i>	North America	USA	Chesapeake Bay, Gulf of Maine	Mclaughlin <i>et al.</i> , 2000; Coss <i>et al.</i> , 2001; Reece <i>et al.</i> , 2008; Marquis <i>et al.</i> , 2020
Perkinsiidae	<i>Perkinsus chesapeaki</i>	Central America	Panama	Caribbean Basin	Pagenkopp Lohan <i>et al.</i> , 2016
Perkinsiidae	<i>Perkinsus chesapeaki</i>	South America	Brazil	Atlantic Ocean	Dantas Neto <i>et al.</i> , 2016
Perkinsiidae	<i>Perkinsus chesapeaki</i>	Europe	France	Mediterranean Sea, Atlantic Ocean	Arzul <i>et al.</i> , 2012
Perkinsiidae	<i>Perkinsus chesapeaki</i>	Asia	India, China	Bay of Bengal, South China Sea	Moss <i>et al.</i> , 2008; Samil <i>et al.</i> , 2012
Perkinsiidae	<i>Perkinsus chesapeaki</i>	Central America	Panama	Caribbean Basin	Pagenkopp Lohan <i>et al.</i> , 2016
Perkinsiidae	<i>Perkinsus chesapeaki</i>	South America	Brazil	Atlantic Ocean	Pinho Ferreira <i>et al.</i> , 2015
Perkinsiidae	<i>Perkinsus behatensis</i>	Asia	China, India	South China Sea, Bay of Bengal	Moss <i>et al.</i> , 2008; Samil <i>et al.</i> , 2012
Perkinsiidae	<i>Perkinsus behatensis</i>	Central America	Panama	Caribbean Basin	Pagenkopp Lohan <i>et al.</i> , 2016
Perkinsiidae	<i>Perkinsus behatensis</i>	South America	Brazil	Atlantic Ocean	Pinho Ferreira <i>et al.</i> , 2015
Perkinsiidae	<i>Perkinsus homshuensis</i>	Asia	Japan, Korea	Pacific Ocean	Dungan and Reece, 2006; Kang <i>et al.</i> , 2016
Perkinsiidae	<i>Perkinsus mediterraneus</i>	Europe	Spain, Italy	Mediterranean Sea	Casas <i>et al.</i> , 2004; Ramilo <i>et al.</i> , 2015
Perkinsiidae	<i>Perkinsus qugwadi</i>	North America	Canada	Gulf of Alaska	Itoh <i>et al.</i> , 2013
Parviluciferaceae	<i>Parvilucifera sinerae</i>	Europe	Spain, France, Portugal	Mediterranean Sea, Atlantic Ocean, English Channel	Figueras <i>et al.</i> , 2008; Garcés and Hoppenrath, 2010; Lepelletier <i>et al.</i> , 2014; Turon <i>et al.</i> , 2015
Parviluciferaceae	<i>Parvilucifera rostrata</i>	Europe	France	English Channel	Lepelletier <i>et al.</i> , 2014
Parviluciferaceae	<i>Parvilucifera corolla</i>	Europe	Spain	Canary Islands, Mediterranean Sea	Reñé <i>et al.</i> , 2017a
Parviluciferaceae	<i>Parvilucifera infectans</i>	Europe	Sweden	North Sea, Baltic Sea	Norén <i>et al.</i> , 1999; Johansson <i>et al.</i> , 2006
Parviluciferaceae	<i>Parvilucifera infectans</i>	Asia	Korea	East China Sea	Jeon <i>et al.</i> , 2018
Parviluciferaceae	<i>Snorkelia prorocentri</i>	North America	Canada	Pacific Ocean	Hoppenrath and Leander, 2009
Parviluciferaceae	<i>Snorkelia prorocentri</i>	Europe	Spain	Mediterranean Sea	Reñé <i>et al.</i> , 2017b
Parviluciferaceae	<i>Dinovorax pyriformis</i>	Europe	Spain	Mediterranean Sea	Reñé <i>et al.</i> , 2017b
Parviluciferaceae	<i>Parvilucifera multicaavata</i>	Asia	Korea	Yellow sea	Jeon and Park, 2020
Parviluciferaceae	<i>Tuberlatum coatsi</i>	Asia	Korea	East China Sea	Jeon and Park, 2019
SPI agent	-	North America	USA	FL, GA, LA, MD, ME, MN, MS, NH, NY,OR, VA, WI	Isidoro-Ayza <i>et al.</i> , 2017; Chambouvet <i>et al.</i> , 2015
Xcellidae	<i>Gadixcellia gadi</i>	Europe	Iceland	Atlantic Ocean	Freeman <i>et al.</i> , 2017
Xcellidae	<i>Gadixcellia</i> sp.	Europe	Iceland	Atlantic Ocean	Freeman <i>et al.</i> , 2017
Xcellidae	<i>Xcellia gobii</i>	Asia	Japan	Pacific Ocean	Freeman <i>et al.</i> , 2017
Xcellidae	<i>Xcellia lemelliphila</i>	Europe	Scotland	North Sea	Freeman <i>et al.</i> , 2017
Xcellidae	<i>Xcellia pleuronecti</i>	Asia	Japan	Pacific Ocean	Freeman <i>et al.</i> , 2017

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Chapitre 1.2 : Diversité environnementale des Perkinsozoa : un premier pas vers la découverte de signatures génétiques encore inconnues dans les écosystèmes pélagiques

Avant-propos

Au cours du **Chapitre 1.1**, nous avons constaté que les Perkinsozoa étaient principalement des parasites à large spectre d'hôtes. Pathogènes pour de nombreuses espèces clés (ingénieurs ou en voie d'extinction), certains de ces membres possèdent un fort potentiel invasif comme dans le cas des Perkinsidae. Cependant, encore aujourd'hui, la plupart des Perkinsozoa ne sont décrits que par leur signature génétique dans différents environnements aquatiques. Alors que cette diversité environnementale énigmatique est soupçonnée de jouer un rôle fonctionnel important dans les écosystèmes dulcicoles, deux études récentes ont révélé une diversité jusqu'alors insoupçonnée au sein de sédiments côtiers marins en Europe et en Antarctique. Cette mise en lumière de nouveaux clusters environnementaux a permis de raviver l'intérêt porté aux Perkinsozoa dans les environnements marins. Au cours de ce **Chapitre 1.2**, nous avons étudié pour la première fois la diversité des Perkinsozoa au sein des communautés picoplanctoniques (0.2 – 3 μm) pélagiques grâce à un jeu de données ADN et ARN issus de l'expédition Malaspina 2010 Circumglobal. Les extractions d'ADN et d'ARN, le séquençage Illumina et le traitement bio-informatique des séquences V4 de l'ADNr/ARNr 18S ont été réalisés en amont par Giner *et al.* (2020) et Obiol *et al.*, (2020). Nous avons donc directement pu identifier les ASVs correspondant aux Perkinsozoa dans ce jeu de données. Ainsi, nous avons étudié leur distribution et leur activité ribosomique à travers 13 stations et sept profondeurs de prélèvement (allant de ~3m à 4 000 m) réparties dans les océans Atlantique, Pacifique et Indien. Ce **Chapitre 1.2** présente donc les premiers pas dans la détection de nouvelles séquences de Perkinsozoa à une l'échelle globale au sein de communautés picoplanctoniques pélagiques (0.2 – 3 μm). Par la suite, cette première ébauche pourrait faire l'objet d'analyses plus approfondies en intégrant d'autres campagnes (e.g. TARA-Oceans, OSD) permettant de mettre en lumière leur diversité et d'émettre des hypothèses quant à leur mode trophique et la nature de leurs interactions au sein de ces écosystèmes.

The Perkinsozoa black-box: a first-step toward an unveiled diversity in the Global Ocean

Abstract

Perkinsozoa (Alveolata) is a lineage of eukaryotic micro-parasites infecting a wide range of host from microalgae, molluscs and fishes in marine environments and amphibians in freshwater environments. Recent molecular surveys of the small sub-unit ribosomal RNA gene (SSU rDNA) sequencing has greatly increased our view of the diversity of this lineage identifying numerous new clusters only represented by their environmental sequences. While a significant diversity and abundance of Perkinsozoa-like phylotype is regularly detected in the water-column of freshwater environments, in marine environments the situation is not so clear cut. Only two recent studies highlighted a hitherto unsuspected wide diversity within coastal sediments in Europeans and in Antarctic fjords samples. These results suggested that these organisms might play a significant unrecognized role in marine sediments as part of 'seed bank' microbial coastal community. However, these studies only focused on coastal environments suggesting that the role and diversity of Perkinsozoa in the Open Ocean is still unknown. Here we test the hypothesis that Perkinsozoa are cryptic under-sampled group in marine water column open in open Ocean environments. Using Illumina sequencing of the V4 region of the 18S rDNA/RNA gene, we investigate the diversity and distribution of these organisms in pelagic picoeukaryotic communities (0.2 – 3 μm) taken from 13 dispatched stations from Atlantic, Pacific and Indian oceans at seven different depths (~3 to 4,000 depth meters) during the Malaspina 2010 Circumnavigation expedition. We detected in the water column the presence of 109 Analysis of Amplicon Sequencing Variant (ASV) Perkinsozoa-like sequences in low abundance (0.002% to 0.64%). Phylogenetic analyses classify these ASV sequences into six major clusters, some of which are closely related to described parasitic organisms (*Snorkelia*, *Dinovorax*, *Tuberlatum*, the Xcellidae or the Perkinsidae. Surprisingly, one cluster (named Cluster A) dominates the global diversity specifically in mesopelagic zone (200 – 1,000 depth meters) with a significant proportion in RNA derived libraries suggesting that this diversity represents ribosomally 'active' and intact cells. Given the phylogenetic host range of Perkinsozoa parasites, these data suggest that Perkinsozoa organisms may play a significant but cryptic role in the mesopelagic zone as symbiotic or free-living heterotrophic organisms that is still needed to be explored.

1. Introduction

Marine microbial eukaryotic communities form a complex interactive network encompassing all kinds of inter-species relationships, e.g. predator-prey and all shades of symbiosis from commensalism to parasitism (Lima-Mendez *et al.*, 2015). Parasitic protists are a key component in ecological succession, food web structure and biogeochemical cycles (e.g. Worden *et al.*, 2015), yet the role and diversity of these organisms are still needed to be described. The Alveolata superphylum includes numerous parasitic lineages as Apicomplexa, Syndiniales (including both marine alveolate groups I and II [MALVI and MALV II]) and Perkinsozoa (syn. Perkinsids, Perkinsea) (Cavalier-Smith, 1993; Chambouvet *et al.*, 2008) (reviewed in Itoiz *et al.* in prep).

The Perkinsozoa lineage is an early branch of Dinoflagellates (Saldarriaga *et al.*, 2003) encompassing today exclusively micro-eukaryotic parasitic species (Itoiz *et al.* in prep). In marine environments, seven genera are today described: *Perkinsus* spp. (Perkinsidae) (Mackin *et al.*, 1950), *Parvilucifera* spp. (Parviluciferaceae) (Jeon *et al.*, 2018), *Dinovorax*, *Snorkelia* and *Tuberlatum* (Reñé *et al.*, 2017; Jeon and Park, 2019), *Xcellia* and *Gadixcellia* (Xcellidae) (Brooks *et al.*, 1969). The Perkinsidae are, to date, composed of seven genetically close species, based on their small subunit (SSU) rDNA encoding gene sequences (> 99% of sequence identity), infecting a wide range of marine molluscs (e.g. oysters, clams, abalone, scallops and cockles). These organisms have been reported from every continent, except Antarctica, and exclusively in coastal marine waters where their target hosts are present (e.g. Villalba *et al.*, 2004; Ruano *et al.*, 2015). The two well-known parasites of this group are *Perkinsus marinus* and *P. olseni*, the aetiological agents of "Dermo" disease and Perkinsosis respectively. These two parasites have been identified as invasive species and responsible of mortality events worldwide with important economic and ecological loss (Andrews, 1996; Siddall *et al.*, 1997; Ruano *et al.*, 2015) leading to list them as notifiable diseases by the World Organization for Animal Health (O.I.E.) (O.I.E. 2019). The Parviluciferaceae and the genus *Dinovorax*, *Snorkelia* and *Tuberlatum* are composed of microalgae parasitic species that are genetically close (> 97% identity based on their SSU rDNA sequences) but formed divergent groups with long-branches within the Perkinsozoa phylum (Reñé *et al.*, 2017; Jeon *et al.*, 2018). These parasites play a key role in micro-algal bloom mitigation including those of noxious species (Alacid *et al.*, 2017). Finally, *Xcellia* and *Gadixcellia* genera have been detected infecting more than 20 fish species belonging to at least five orders of teleosts (Freeman *et al.*, 2017). Although

these two genera show unusually high genetic differences based on their SSU rDNA sequences (74.9 %), all described species are fish parasites representing two independent radiations within the Perkinsozoa phylum (Freeman *et al.*, 2017). Conversely, only one group was described in freshwater environments: the “Severe Perkinsea Infection” (SPI) agent infecting frog tadpole life stage (Davis *et al.*, 2007; Isidoro-Ayza *et al.*, 2017; Chambouvet *et al.*, 2020). This parasite is now identified as an emerging threat responsible for mortality events across the U.S.A. (Isidoro-Ayza *et al.*, 2017, 2019). Hence, all Perkinsozoa described species are parasites whose represent a major ecological and economical threats because of their invasive (Ford, 1996; Vilas *et al.*, 2011), pathogenic (leading to mortality event) (Ruano and Cachola, 1986; Park and Choi, 2001) and high phenotypic plasticity potential (reviewed in Itoiz *et al.* in prep) which raises new scientific questions about the management of emerging infectious diseases in a context of globalisation and climate change.

Environmental DNA (eDNA) analyses, in freshwater ecosystems, have demonstrated that the described group of SPI agents is just the tip of the iceberg. The Perkinsozoa-like sequences are far more diverse and abundant with whole clusters composed only of environmental sequences suggesting an important but neglected ecological role in food web dynamic (Lepère *et al.*, 2008; Mangot *et al.*, 2011; Jobard *et al.*, 2020). However, in marine environments, these sequences often represent less than 1 % of relative reads abundance in environmental DNA/RNA (eDNA/RNA) datasets (Chambouvet *et al.*, 2014; De Vargas *et al.*, 2015). Recent environmental surveys revealed that the wide diversity of Perkinsozoa-like sequences was retrieved in marine coastal sediments with a significant proportion detected in RNA-derived libraries suggesting that these cells are ribosomally 'active' and possibly part of the 'seed bank' microbial communities (Chambouvet *et al.*, 2014; Cleary and Durbin, 2016). However, these few studies only focused on specific coastal European ecosystems and Antarctic fjords suggesting that the role and diversity of Perkinsozoa in the Open Ocean is still unknown. Considering that the main groups described are from marine ecosystems, the lack of specific studies on their diversity in marine environments represents an important scientific question that need to be urgently answered considering these putative emerging infectious agents responsible for mortality events worldwide.

In this paper, we explore the Perkinsozoa diversity in picoeukaryotic communities from the water column using Illumina sequencing of the V4 region of the 18S rDNA/RNA genes derived from the recent study of Giner *et al.* (2020). Picoeukaryotic communities (< 3 µm) were

sampled during the Malaspina 2010 Circumnavigation expedition (Duarte, 2015). We analysed samples from seven depths (from surface to 4 000 depth meters) in 13 stations encompassing the Atlantic, Indian and Pacific Oceans. In this study, we will investigate vertical changes of the Perkinsozoa diversity, abundance, distribution and relative activity in communities of picoeukaryotes across the world's Ocean and test the hypothesis that Perkinsozoa are hitherto under sampled group in marine environments.

2. Material & Methods

2.1. Sampling, extraction and sequencing

During the Malaspina 2010 Circumnavigation expedition (December 2010–July 2011), a total of 91 water samples were collected in 13 stations distributed across the world's major oceans: four in the Pacific Ocean, six in the Atlantic Ocean and three in the Indian Ocean (Figure S1). Each station was sampled at seven different depths including surface (3 depth meters), deep chlorophyll maximum (DCM), mesopelagic (200–1 000 depth meters) and bathypelagic (1 000–4 000 depth meters) waters. For each sample, about 12 L of seawater was prefiltered through a 200 µm nylon mesh following filtration through a 20 µm then 3 and 0.2 µm pore-size polycarbonate filters of 142 mm diameter (Isopore, Millipore) (Giner *et al.*, 2020). The extraction, PCR and sequencing protocols were detailed previously in Giner *et al.* (2020). Briefly, filters of 0.2 µm (containing the 0.2–3 µm size fraction) were cryo-grinded and DNA and RNA samples were extracted simultaneously using the Nucleospin RNA kit (Macherey-Nagel) and the NucleoSpin RNA/DNA Buffer Set (Macherey-Nagel) protocols. Residual DNA and RNA were removed using Turbo DNA-free kit (Applied Biosystems) and RNA were reverse transcribed to cDNA using the SuperScript III reverse Transcriptase (Invitrogen) and random hexamers (Giner *et al.*, 2020). PCR amplifications were realised with the eukaryotic universal primers TAREukFWD1 (5'-CCAGCA(G/C)C(C/T)GCGG-TAATTCC-3') and TAREukREV3 (5'-ACTTTCGTTCTTGAT(C/T)(A/G)A-3') (Stoeck *et al.*, 2010). Picoeukaryotic diversity was assessed by amplicon sequencing of the V4 region of the 18 S rDNA/RNA gene (~380 bp) using the Illumina MiSeq platform and paired-end reads (2 × 250 bp).

2.2. Amplicon data processing

In V4 datasets (rDNA/RNA), reads were processed using the pipeline set up by Obiol *et al.* (2020)⁵ as follows: raw reads (2x250 bp) were trimmed for amplification primers with Cutadapt v1.16 (Martin, 2011) and processed using the software package dada2 v1.12.1 (Callahan *et al.*, 2016) with the parameters truncLen 210,220 and maxEE 6,8. The table of amplicon sequence variants (ASV) with their abundance in each sample was obtained. Samples contained 51 421 reads on average (standard error 4.9). Then, the table was prefiltered: singletons, ASVs with less than 300 bp, ASVs appearing only in one sample with less than ten reads and samples with less than 500 reads (nine in total) were discarded. The V4 ASVs were taxonomically classified using EukaryotesV4 database (total of 25 849 sequences with 43% derived from environmental sequences) built by Obiol *et al.* (2020). ASVs classified as Archaea, Bacteria, Charophyta, Ulvophyceae and Metazoa were discarded (1.3 % of reads).

2.3. Phylogenetic analysis

Alveolata SSU rDNA alignment of 60 taxa sequences from all the major alveolate lineages (Apicomplexa, Dinoflagellata, Perkinsozoa, ciliates, MALVs) was recovered from Chambouvet *et al.* (2014). Total of 110 putative Perkinsea ASVs were aligned to the Alveolata reference alignment using MAFFT v.7.402 with iterative refinement methods Q-INS-i (<http://mafft.cbrc.jp/>) to confirm their taxonomic assignation to Perkinsozoa lineages (Figure S2). The alignment was then improved manually using Seaview v. 3.2 (Gouy *et al.*, 2010) and ambiguously aligned characters were masked. A preliminary tree was used to identify long-branch or highly novel sequences that could be identified as chimeric sequences as previously described by (Chambouvet *et al.*, 2014).

A reference Perkinsozoa SSU rDNA multiple sequence alignment from 216 taxa including Perkinsozoa SSU rDNA sequences (n= 107 sequences from both environmental and cultured organisms) available from non-redundant (nr) nucleotide NCBI database (accessed on October 2020) was obtained. A selection of eight sequences belonging to dinoflagellates and syndiniales were used as outgroup. The Perkinsozoa ASVs were aligned using Q-INS-i iterative refinement methods based on the structural reference alignment (Chambouvet *et al.*, 2014) available from

⁵ Aleix Obiol (Institut de Ciències del Mar, Barcelona) realised the amplicon data processing on the V4 SSU dataset used in this study.

MAFFT v.7.402 (Katoh *et al.*, 2019). The Perkinsozoa SSU alignment was improved manually as mentioned before. The final alignment resulted of 216 taxa with 369 character alignment positions including solely flanking and V4 regions of the SSU sequences. Prior to phylogenetic analysis, the most appropriate nucleotide substitution model was assessed using Modelgenerator v0.85.1 (Keane *et al.*, 2010). A GTR+ Γ substitution model was selected with the α parameter for the Gamma distributions of 0.48 with eight rate categories. The ML phylogenetic tree was reconstructed using RAxML v. 8.2.12 (Stamatakis, 2014) on the CIPRES science gateway (available at <http://www.phylo.org/>). Bootstrap support values were estimated using RAxML v8.2.12 with 1000 pseudoreplicates. Bayesian posterior probabilities were calculated using MrBayes v3.2.6 on the CIPRES science gateway (available at <http://www.phylo.org/>). The model GTR + Γ (lset, nst=6 rates=gamma) was selected and the covarion parameter was selected. The chains were run for 2 000 000 generations with two replicate tree searches both with 4 MCMC chains with heat parameter of 2. Trees were sampled every 200 generations. In both analyses, the MCMC searches had converged within the first 25% of the generations sampled, as such the first quarter of the search results were discarded (as the burnin). The consensus topologies and posterior probabilities of each node were then calculated from the remaining trees. Perkinsozoa ASVs were grouped into ‘clusters’ on the basis of two restrictive criteria: 1) coherent topology support between ML and Bayesian phylogeny and 2) ASVs are present at least in two distinct samples.

2.4. Statistical analysis

Relative abundance of each main clusters (Table 1) belonging to Perkinsozoa were computed with R v. 4.0.3 using RStudio interface (Team, 2013) and their global distribution using QGIS 3.4.5-Madeira software (QGIS.org, 2020). Similarity between sequences (% of identity) were calculated using the software Geneious Prime (Kearse *et al.*, 2012). We investigated the ribosomal activity for each cluster using RNA:DNA ratios as defined in Giner *et al.* (2020). Briefly, ratios >1 indicated ribosomally “hyperactive” taxa, ratios <1 indicated ribosomally “hypoactive” taxa, while ratios ~ 1 pointed to taxa with “average” activity levels. Ratios were plotted using ggplot2 package (Wickham, 2016) on Rstudio software (Team, 2013).

Table 1. Reads number and relative abundances of Perkinsozoa in samples from RNA and DNA Malaspina 2010 picoplanktonic dataset.

Station	Sample	Matrix	Total of reads	Total Perkinsozoa reads	Relative abundance of Perkinsozoa (%)
6	R74	RNA	66030	3	0,005%
6	R76	RNA	198643	44	0,022%
6	R78	RNA	49246	45	0,091%
18	R279	RNA	104901	49	0,047%
30	R522	RNA	69217	82	0,118%
30	R524	RNA	116293	15	0,013%
39	R672	RNA	105029	2	0,002%
39	R676	RNA	41664	19	0,046%
39	R678	RNA	175801	194	0,110%
49	R868	RNA	118561	15	0,013%
49	R870	RNA	51 761	6	0,012%
49	R872	RNA	12 6527	89	0,070%
55	R1005	RNA	57869	9	0,016%
55	R1007	RNA	112545	15	0,013%
63	R1158	RNA	63903	3	0,005%
92	R1168	RNA	47788	5	0,010%
92	R1660	RNA	34562	2	0,006%
92	R1664	RNA	95572	443	0,464%
92	R1666	RNA	45064	287	0,637%
92	R1668	RNA	45074	118	0,262%
96	R1764	RNA	47136	10	0,021%
96	R1766	RNA	45443	43	0,095%
96	R1768	RNA	40688	65	0,160%
110	R2021	RNA	63807	3	0,005%
110	R2025	RNA	38622	47	0,122%
110	R2027	RNA	32853	167	0,508%
120	R2029	RNA	35951	2	0,006%
120	R2225	RNA	33491	14	0,042%
128	R2410	RNA	139379	4	0,003%
128	R2416	RNA	41282	64	0,155%
128	R2418	RNA	150034	312	0,208%
141	R2799	RNA	96095	9	0,009%
141	R2801	RNA	89626	87	0,097%
6	D76	DNA	118937	26	0,022%
6	D78	DNA	191171	92	0,048%
18	D277	DNA	93920	2	0,002%
18	D279	DNA	168075	148	0,088%
30	D518	DNA	175287	59	0,034%
30	D522	DNA	184041	124	0,067%
30	D524	DNA	162825	13	0,008%
39	D672	DNA	105272	136	0,129%
39	D678	DNA	70238	10	0,014%
49	D868	DNA	116049	39	0,034%
49	D870	DNA	69376	18	0,026%
49	D872	DNA	55961	16	0,029%
55	D1005	DNA	76549	46	0,060%
63	D1158	DNA	50353	37	0,073%
63	D1168	DNA	48135	7	0,015%
92	D1664	DNA	34928	13	0,037%
92	D1666	DNA	51270	10	0,020%
92	D1668	DNA	106356	16	0,015%
96	D1766	DNA	80420	4	0,005%
96	D1768	DNA	107360	13	0,012%
110	D2027	DNA	45327	42	0,093%
120	D2221	DNA	131498	14	0,011%
128	D2410	DNA	62673	99	0,158%
128	D2414	DNA	117125	89	0,076%
128	D2416	DNA	73673	31	0,042%

3. Results and discussion

3.1. Perkinsozoa in the Malaspina 2010 picoeukaryotic datasets

The rDNA/RNA V4 final table contained 28 518 ASVs and 15×10^6 reads. In this global picoeukaryotic Malaspina dataset, solely 0.4 % (110 ASVs) of the ASV diversity was assigned to Perkinsozoa group. Phylogenetic analysis demonstrated that 109 ASVs representing 3 376 reads were correctly assigned to Perkinsozoa whereas one ASV (ASV_42439) was badly assigned and branched within Dinoflagellate and MALV (Figure S2; Table S1). The sequence ASV_42439 was discarded from analyses.

Although of Perkinsozoa genetic signatures were revealed in marine sediments (López-García *et al.*, 2003; Chambouvet *et al.*, 2014; Cleary and Durbin, 2016) and freshwater lakes (Lepère *et al.*, 2008; Mangot *et al.*, 2011; Jobard *et al.*, 2020), there is few specific investigations in marine pelagic ecosystems because of their rare abundance and their reduced diversity, mainly represented by *Parvilucifera* genus, parasites of dinoflagellates (Chambouvet *et al.*, 2014). In this study, a total of 109 ASVs confirmed their genetic affiliation to the Perkinsozoa lineage. In the Malaspina 2010 RNA dataset, Perkinsozoa reads represent between 0.006 % to 0.637 % of the total of reads per sample. In the Malaspina 2010 DNA dataset, Perkinsozoa reads represent between 0.002 % to 0.158 %. Perkinsozoa were detected in low abundance environmental sediment samples as mentioned in 2014 by Chambouvet *et al.* (0.006% to 0.244% using 454 sequencing effort). In some stations, relative abundances of Perkinsozoa ASVs are higher than 0.01 %, which is the threshold determined by Logares *et al.*, (2014) to identify rare taxa, suggesting that their role in pelagic ecosystems may be more important than expected (Table 1).

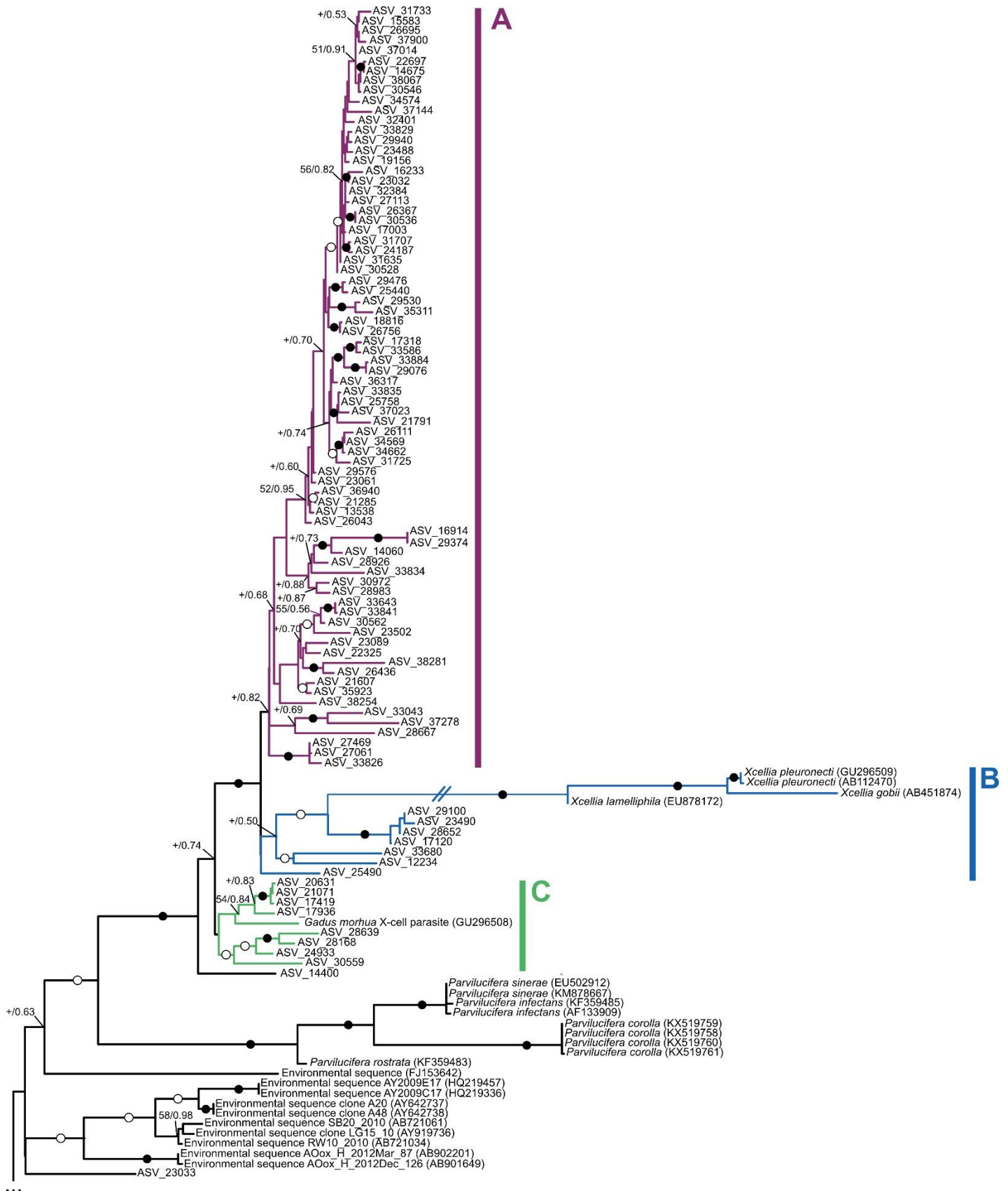
3.2. Diversity within marine Perkinsozoa

The Perkinsozoa phylogeny, based on the V4 SSU rDNA region, demonstrated major groups of marine species already described in the previous studies. As previously described in Freeman *et al.* (2017), the Xcellidae were divided into the two main genera *Xcellia* and *Gadixcellia* sharing 79 % of sequence similarity and supported by consistent tree topologies. Similarly, three different genera of dinoflagellate parasites, *Snorkelia*, *Dinovorax* and *Tuberlatum*, were

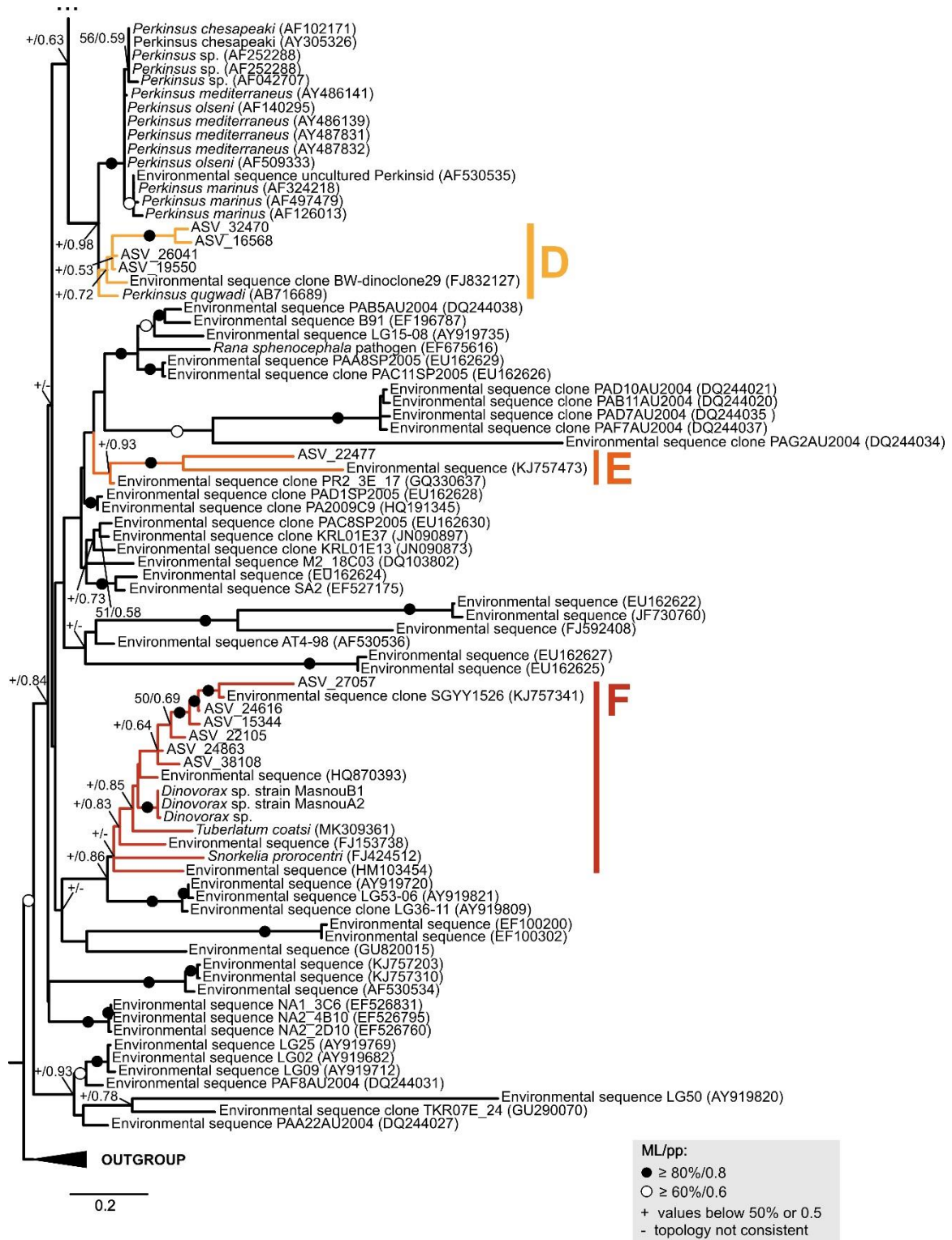
grouped together with consistent phylogeny. Perkinsidae (99% of sequence identity) and Parviluciferaceae (86 % of sequence identity) were clearly identified and supported by high bootstrap values and posterior probability (>80 %/0.8). ASVs included in this phylogeny can be directly branched to described (e.g. Xcellidae, Perkinsidae) or environmental clade but can also be more independent (Figure 1).

Using this approach, we identified six phylogenetic clusters labelled as cluster A to F (Figure 1). Cluster A represented a big group of undescribed marine diversity (76 ASVs) sharing 81 to 100 % of sequence identity and did not match any referenced sequences on NCBI Genbank database (accessed in October 2020). Cluster B (6 ASVs) and C (9 ASVs) were more related to fish parasites *Xcellia* (56 – 62 %) (GU296509, AB112470, AB451874 and EU8787172) and *Gadixcellia* (83 – 90 %) (GU296508) respectively. Cluster B harboured a high genetic divergence explaining the long branch formed by *Xcellia* sequences. Cluster D (4 ASVs) was composed of the scallop parasite, *Perkinsus qugwadi*, sequence (AB716689) and the environmental sequence (FJ832127) at the basal position of the *Perkinsus* cluster. Cluster E (one ASV) was related to marine environmental sequence KJ757473 and GQ3306367 (82 % of sequence identity). Finally, the cluster F (6 ASVs) was more related to the genera *Dinovorax*, *Snorkelia* and *Tuberlatum* composed of described dinoflagellate parasites (MK309361, FJ424512) and the environmental sequences (KJ757341, HQ870393, FJ153738 and HM103454) sharing 88 to 94 % of sequence identity. This late cluster, sharing 94 % of sequence identity between described species (*Dinovorax* sp., *Tuberlatum coatsi* and *Snorkelia* sp.) suggested that ASV sequences could be putative parasites with close host range and functional role in marine environments. Additionally, two unique ASV sequences were not clustered using our classification criteria. Globally, *Perkinsus* genus had a high conservative V4 region between different species (99 % of sequence identity) contrary to *Xcellia* (89 % of sequence identity) and *Parvilucifera* (86 % sequence identity) genera. If these thresholds of sequence identity are consistent to determine Perkinsozoa genera, it suggests that clusters A – F are composed of several unknown phylogenetic levels with distinct biological and/or ecological traits, i.e. other parasitic taxa with diverse host ranges or free living heterotrophs.

Chapitre 1.2 : Diversité environnementale des Perkinsozoa dans les écosystèmes pélagiques

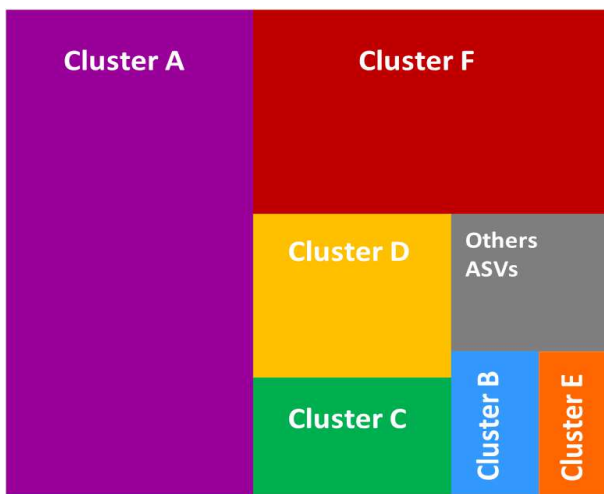


Chapitre 1.2 : Diversité environnementale des Perkinsozoa dans les écosystèmes pélagiques



◀ **Figure 1. Perkinsozoa V4 18S rRNA phylogeny including ASV V4 sequences from Malaspina 2010 Circumglobal Expedition sampling.** RAxML phylogeny of Perkinsozoa V4 18S rRNA sequences based on the analysis of 216 sequences including Perkinsozoa sequences from Genbank (n= 107) and ASV sequences (n= 109) from Malaspina 2010 Circumglobal Expedition sampling. The phylogeny was calculated from 216 taxa and 369 characters alignment position. ML bootstrap values (1 000 replicates) and Bayesian posterior probability (pp; 2 000 000 generations, GTR+ Γ model) were notated using the following convention: support values are summarized by \blacktriangle when $\geq 80\%/0.8$ and white circle when it is not the case but values $\geq 60\%/0.6$. When support value is $<60\%/0.6$ but topology is consistent, the support value is summarized by a '+' and conversely by a '-' if the topology is not consistent. Eight sequences of dinoflagellates and syndiniales were used as an outgroup. Genbank accession numbers were referenced in brackets.

DNA Perkinsozoa ASVs



RNA Perkinsozoa ASVs

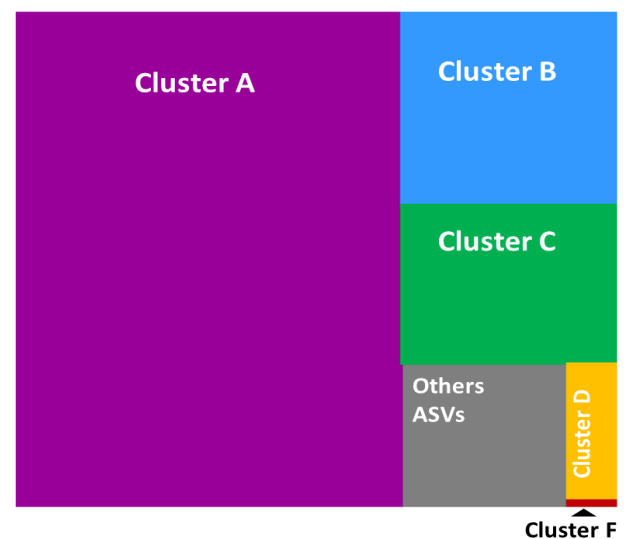
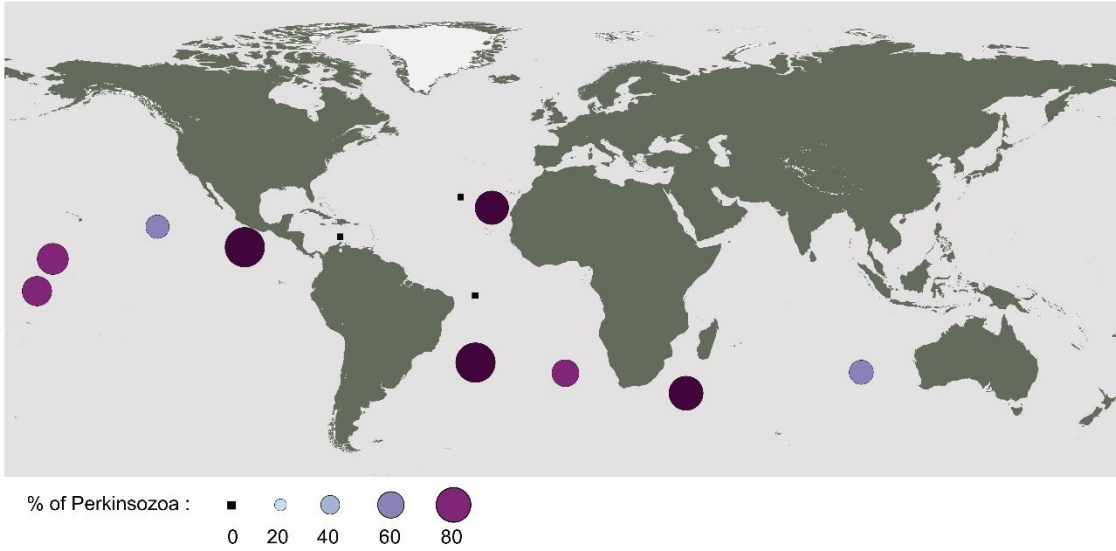


Figure 2. Treemaps of the number of reads of Perkinsozoa depending on the six main clusters from the whole DNA and RNA Malaspina 2010 datasets. All Perkinsozoa ASVs were merged (Table 1; Table S1).

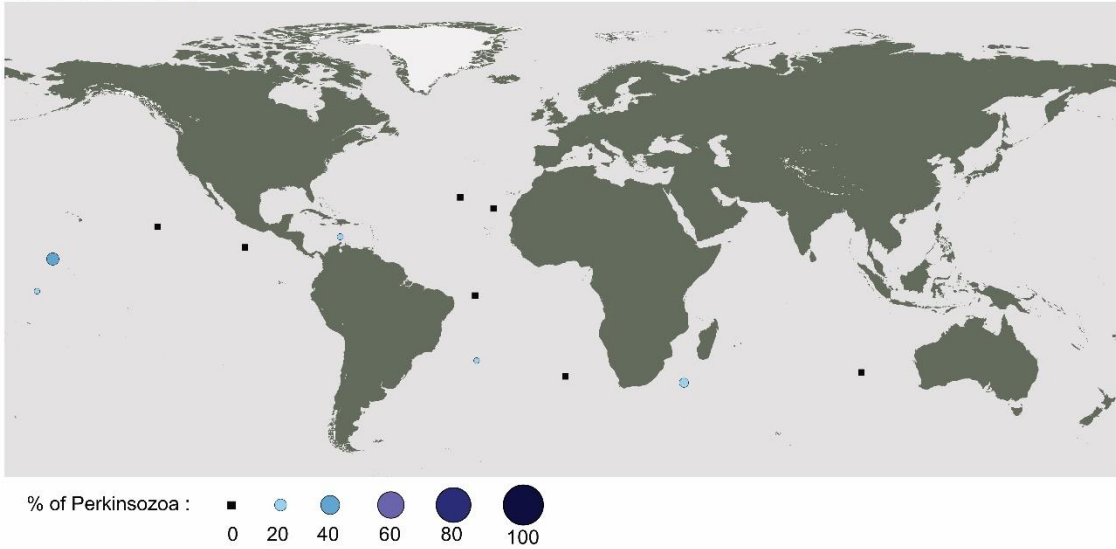
Although, we could have chosen more stringent criteria (e.g. bootstraps > 60/0.6) and focused on terminal groups within the Perkinsozoa phylogeny to discriminate more precisely clusters diversity, some clusters remained too genetically divergent (cluster B) or are not affiliated to a published sequence (cluster A). Hence, the functional and biological role of its genetic sequences remains entirely unknown which make difficult subdivision in smaller groups. Above all, the aim of the present study was to assess an overview of Perkinsozoa, we thus grouped ASVs following these six main clusters to investigate their global distribution and activity within the different samples.

In the Malaspina 2010 picoplanktonic dataset, the main Perkinsozoa abundance and diversity was dominated by the cluster A, 40 % of DNA reads 64 % of RNA (cDNA) reads (Figure 2). Clusters B, C, D and F showed clear differences between DNA and RNA Malaspina 2010 datasets suggesting that some clades could be less active/dead organisms or represented by solely by free DNA as it is suggested for cluster E (Figure 2).

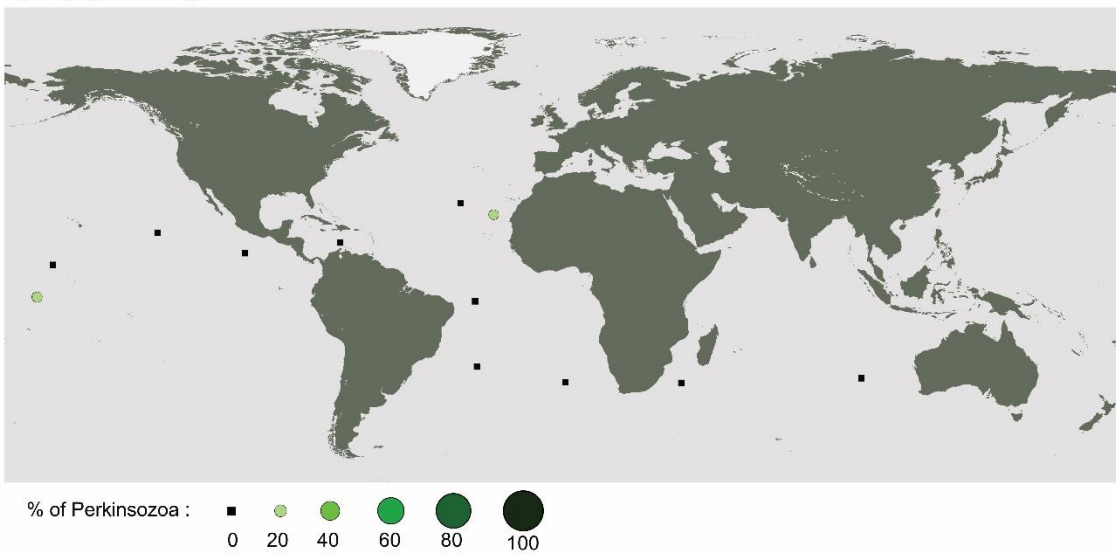
CLUSTER A



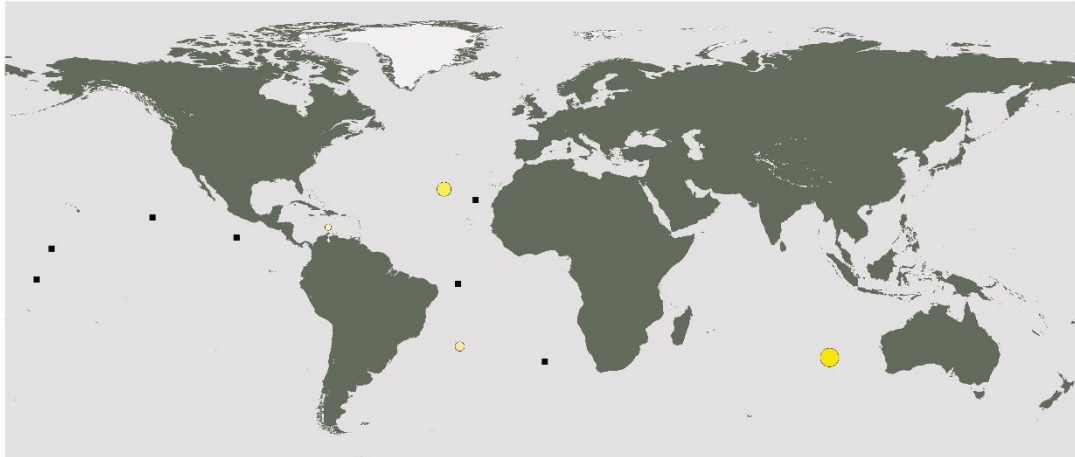
CLUSTER B



CLUSTER C

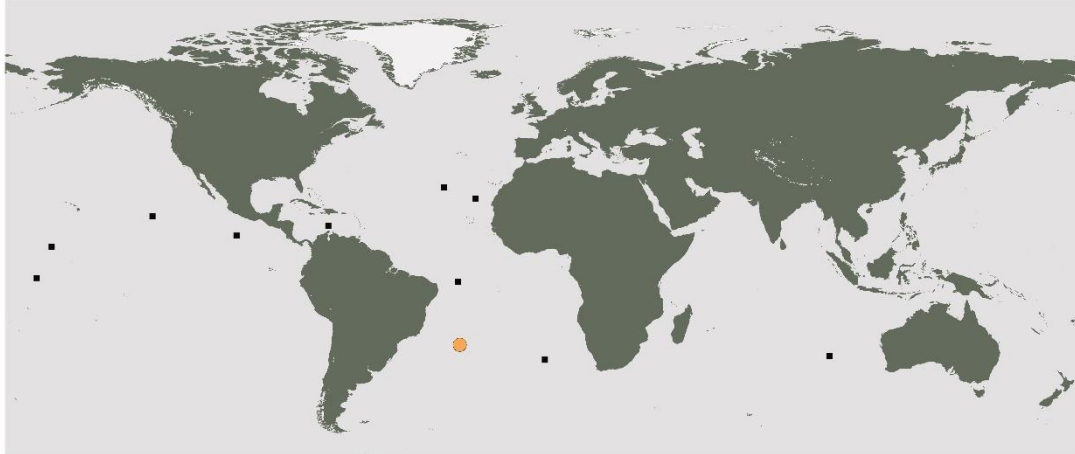


CLUSTER D



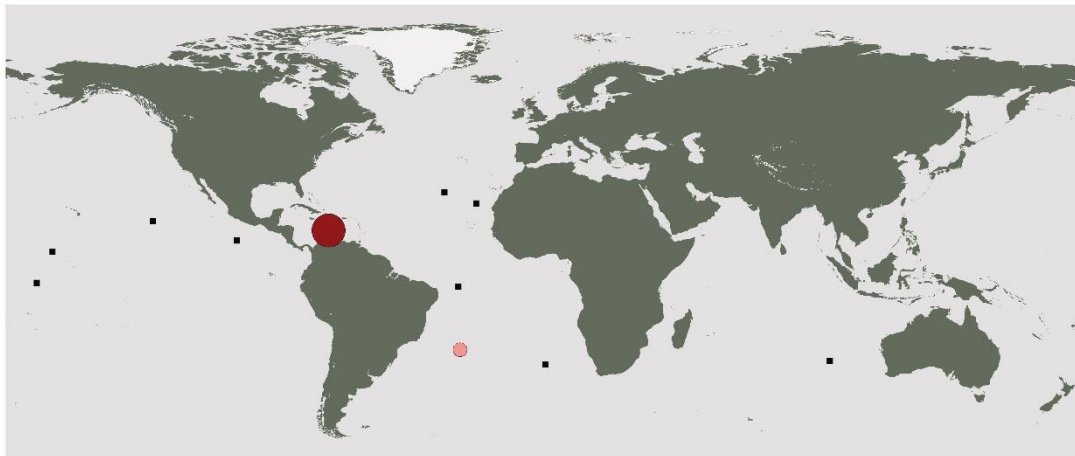
% of Perkinsozoa : ■ ● ● ● ● ●
0 20 40 60 80 100

CLUSTER E



% of Perkinsozoa : ■ ● ● ● ● ●
0 20 40 60 80 100

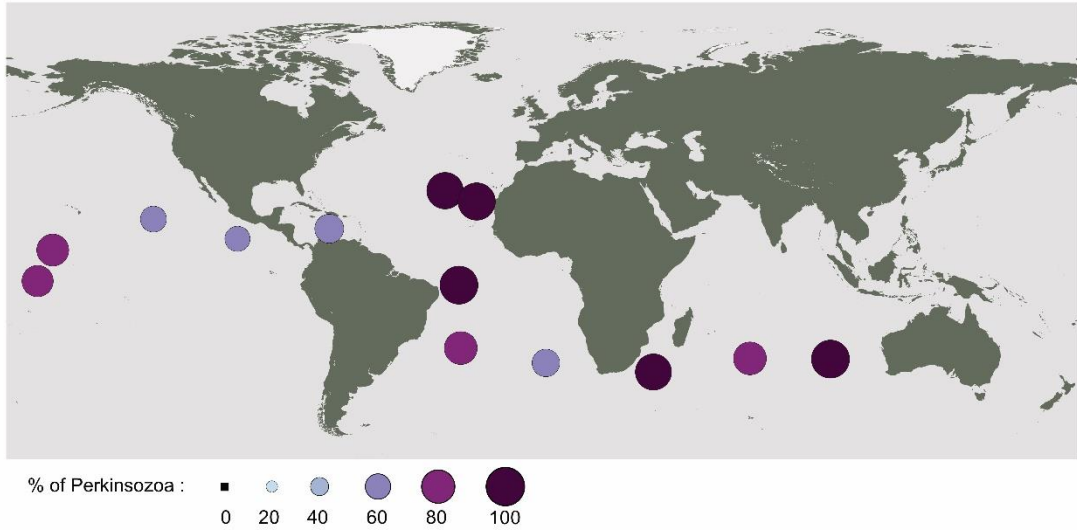
CLUSTER F



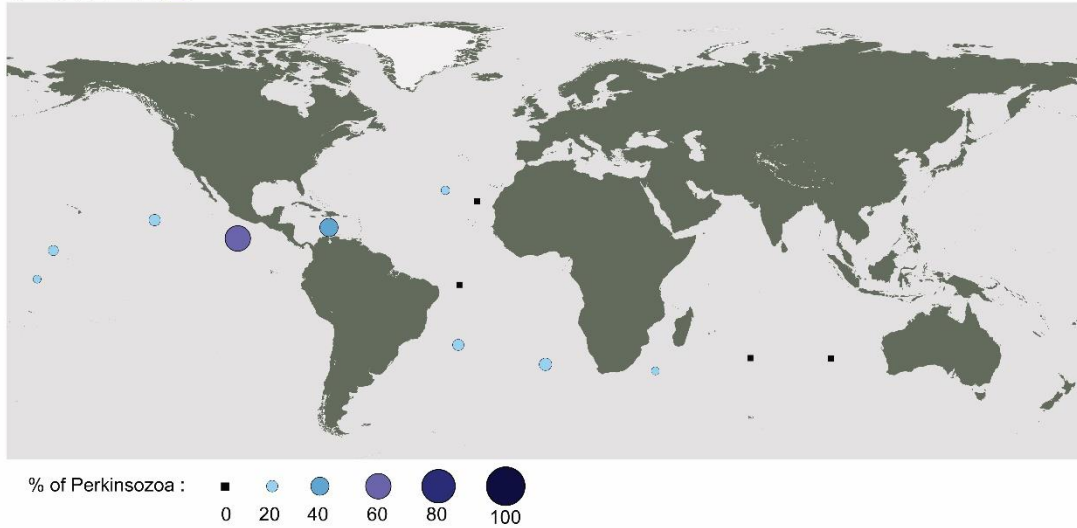
% of Perkinsozoa : ■ ● ● ● ● ●
0 20 40 60 80 100

◀ **Figure 3.** Distribution of the six main clusters of Perkinsozoa ASVs from the DNA Malaspina 2010 picoplanktonic dataset. ASVs of a same cluster from an identical station were pooled together. Circle corresponds to the percentage of ASV reads relative to the total number of Perkinsozoa reads sequenced in the station. Non-clustered ASVs were not represented.

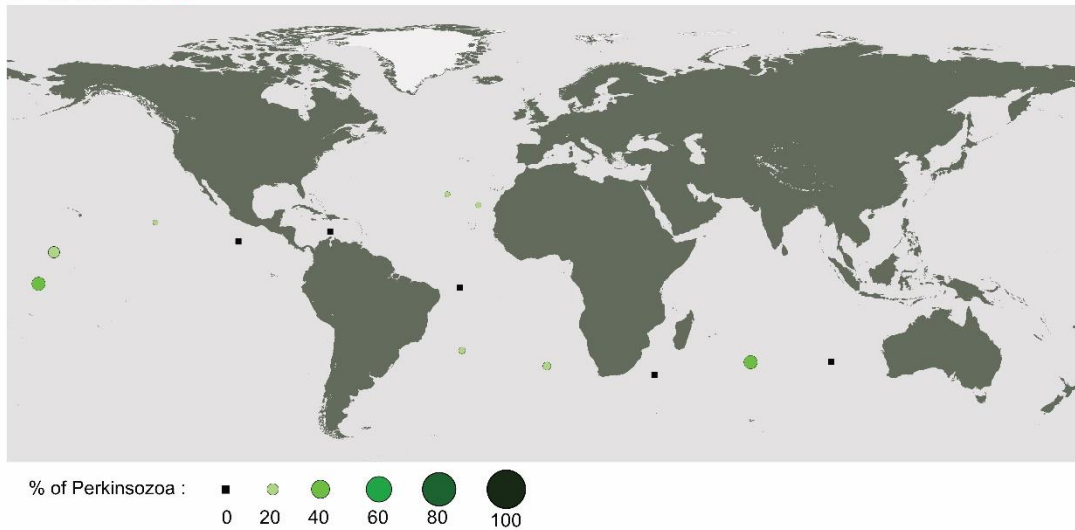
CLUSTER A



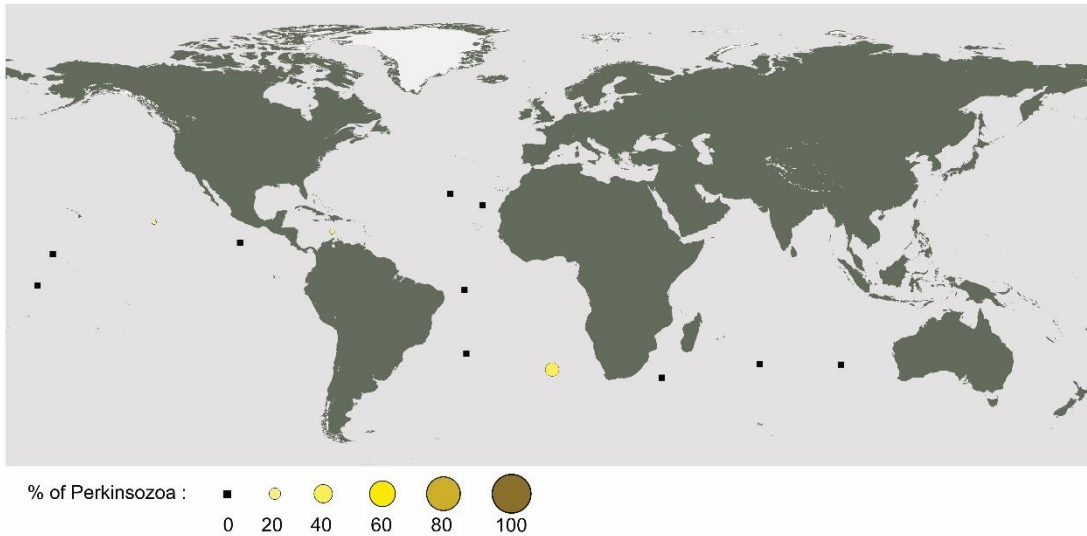
CLUSTER B



CLUSTER C



CLUSTER D



CLUSTER F

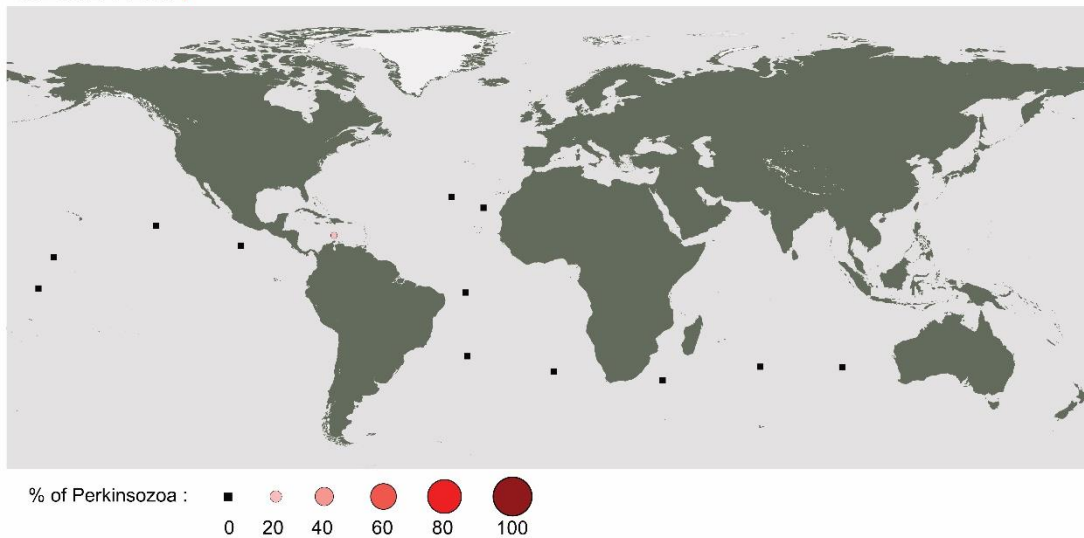


Figure 4. Distribution of the five main clusters of Perkinsozoa ASVs from the RNA Malaspina 2010 picoplanktonic dataset. ASVs of a same cluster from an identical station were pooled together. Circles correspond to the percentage of ASV reads relative to the total number of Perkinsozoa reads sequenced in the station. Non-clustered ASVs were not represented.

3.3. Distribution of the Perkinsozoa environmental clades

Cluster A was widespread and highly abundant in all oceans representing up to 85 % of DNA and 97 % RNA total Perkinsozoa reads (Figure 3 & 4). In contrast, clusters B and C were also distributed in the three Oceans considering their rRNA signatures but in lower abundance (40 % and 23 % respectively) (Figure 4). Occurrence of their rDNA signatures were detected in solely five stations for cluster B and two stations for cluster C (Figure 3). Clusters D and F occurred solely in the Atlantic Ocean and Indian Ocean (exclusively for Cluster D) and were totally absent from stations of the Pacific Ocean (Figure 3 & 4). DNA and RNA signatures of these two clusters in the Station 128, close to the Panama Channel, could be relevant with previous environmental study finding *Perkinsus* and *Parvilucifera* genetic signatures in this geographical area (Lohan *et al.*, 2016). Cluster E was totally absent from RNA dataset and its DNA signature occurred exclusively in station 39 in the Atlantic Ocean (Figure 3 & 4). In this study, no clear large-scale pattern has been determined and no clusters seemed to be endemic from a specific geographical area. However, it is clear that the three Oceans are dominated by cluster A abundances and distribution in both rDNA and rRNA derived libraries. Clusters B and C are also well represented in rRNA samples and have the characteristic of being close to the fish parasites *Xcellia* and *Gadixcellia*. In view of the genetic divergence within the genus *Xcellia* (89 % of sequence identity), we could hypothesise that close ASVs could share similar host range which allow a facilitating dissemination by fish migration or nursery area. Indeed, a highly migratory lifecycle, like for salmon, could be a source of micro-parasite translocations from one environment to another (reviewed in Miller *et al.*, 2014). Or, these clusters could be directly related to fish populations inhabiting the mesopelagic zone which represent an important biomass of 14 to 19.5 gigatonnes of potential hosts (Irigoien *et al.*, 2014).

3.4. Ribosomally active environmental clades in mesopelagic waters

Even if no specific geographical pattern was observed, a vertical distribution of these Perkinsozoa-like ASVs was really clear. ASVs relative abundances were analysed from seven depths from the surface (~3 depth meters) to the bathypelagic zone (max. 4 000 depth meters). Surprisingly all clusters are completely absent from the surface and DCM depth (Figure 5 & 6) including cluster F which branched close to dinoflagellate parasites (e.g. *Dinovorax* spp., *Snorkelia* spp. and *Tuberlatum* spp.). Clusters A, B and C occurred in mesopelagic waters whereas clusters D, E and F occurred mainly in bathypelagic waters (Figure 5). Moreover,

abundances in the bathypelagic zone are mainly found in the dataset from DNA-derived libraries suggesting that diversity of clusters D, E and F could be hypoactive (e.g senescent cells) or represented by free DNA. These observations were strengthened by the analysis of RNA:DNA ratios (Figure 6). Indeed, cluster A, mainly found in the mesopelagic zone – between 400 and 700 m depth - of the Atlantic and Pacific Oceans, was represented by “hyperactive” organisms (ratios > 1) (Figure 6). This last cluster and cluster B and C had high occurrences in rRNA templates (Figure 6). ASVs detected in rRNA templates should be explained as an increase of cellular activities of some rare taxa that were not identified in the rDNA datasets (Pochon *et al.*, 2017). Finally, the activity of these three clusters in the picoplankton could be represented as putative active and small life stages. These results are consistent with the description of *Perkinsus* and *Parvilucifera* life cycle. Indeed, direct life cycle of *Perkinsus* and *Parvilucifera* species includes a free life stage, the zoospores (1-2 μm), which can be disseminated in the water column to infect new hosts (Villalba *et al.*, 2004; Alacid *et al.*, 2015). While the hypotheses about clusters B and C are oriented towards a functional role close to the *Xcellia* and *Gadixcellia* parasites as mentioned above, cluster A remains enigmatic as no close publically available sequence was affiliated from NCBI database. Taken all together, cluster A might represent an under sampled but important group in mesopelagic picoplanktonic communities which are mainly composed of Ciliates, Dinoflagellates, Labyrinthulomycetes, Cercozoans and some MALV groups (Giner *et al.*, 2020) . Unfortunately, it is not possible to resolve whether this diversity hides flagellate parasitic and/or free-living heterotrophic organisms or a methodological artefact corresponding to broken cells from upper size fractions.

Finally, clusters D, E and F were mainly characterised by their genetic signatures in the rDNA-derived libraries (Figure 6) suggesting a sinking stabilised eDNA or sinking senescent and/or lysate cells. Furthermore, these data may also provide from a methodological artefact produced by the filtration procedure which amplify dissolved DNA from hypothetical larger organisms or dead cells (Massana, 2011; Barnes and Turner, 2016). These methodological artefact hypotheses are coherent for clusters D and E because i) these clusters are only retrieved from rDNA derived libraries, ii) described dinoflagellates and mollusc parasitic species affiliated to these clusters are, up today, only detected in coastal environments (Villalba *et al.*, 2004; Reñé *et al.*, 2017; Jeon and Park, 2019).

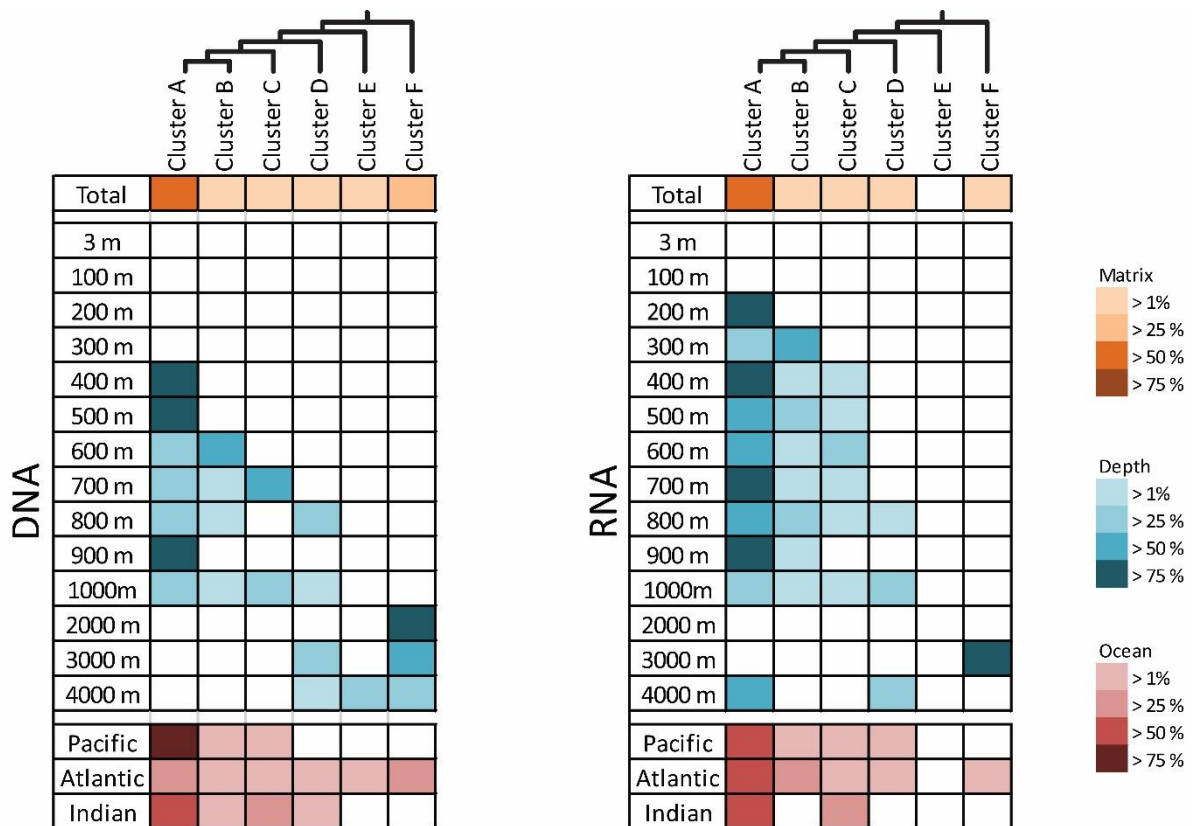
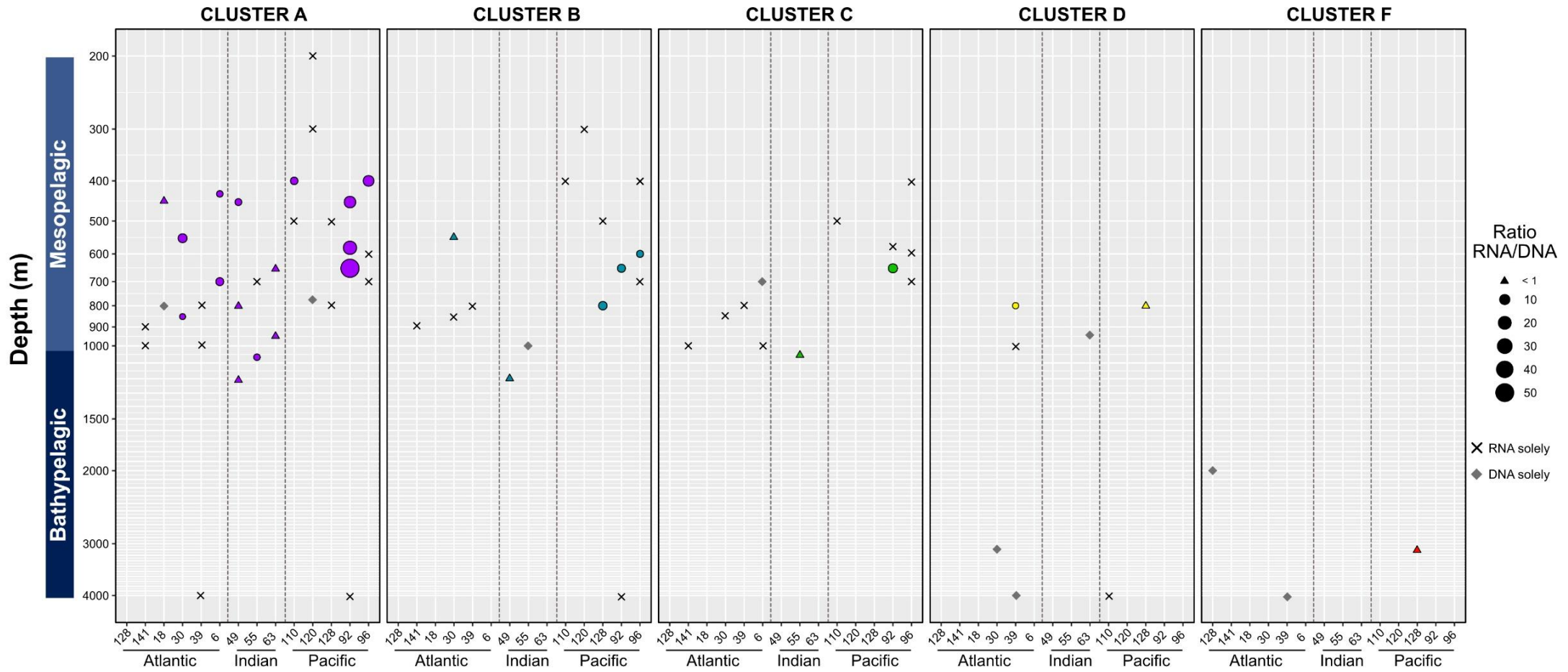


Figure 5. DNA and RNA matrices of relative abundances of the six Perkinsozoa-like clusters. Relative abundances were calculated based on total Perkinsozoa reads per samples. Orange scale corresponded to relative abundances in the whole dataset. Blue scale corresponded to global relative abundances at each depth. Red scale corresponded to global relative abundances in each ocean.

Figure 6. RNA: DNA ratios for Perkinsozoa-like clusters along vertical profile of each station. Ratios >1, indicating metabolically “hyperactive” taxa, were represented by coloured circles. Ratios <1, indicating metabolically “hypoactive” taxa, were represented by coloured triangle. RNA genetic signatures with no equivalent in DNA are represented with a cross. Conversely, DNA genetic signatures with no equivalent in RNA are represented with a grey diamond.



4. Conclusion

In the picoplankton from the open ocean, Perkinsozoa group is diverse, low abundance and very localised in the water column. This putative Perkinsozoa genetic signatures are ribosomally active in the mesopelagic waters from 200 to 1 000 depth meters while they are totally absent of the photic zone. Genetic signatures found in the bathypelagic waters are represented by dominance of DNA samples resulting in hypoactive, dying cells or extracellular DNA exported to deep waters. Clusters D, E and F are more specific to coastal ecosystems and therefore absent from pelagic environments. Conversely, clusters A, B and C emerged as diverse, well represented and hyperactive in the 0.2 – 3 μm size fraction from the mesopelagic zone. Hypothetically, these clusters may be putatively affiliated either to a parasitic flagellate life stages related to zoospores (1 - 2 μm) or a small free-living heterotrophs. While phylogenetic analyses revealed that clusters B and C encompass *Xcellia* and *Gadixcellia* parasites, any sequences retrieved in NCBI databases have been successfully assigned to the cluster A. However, the recurrence of this cluster in different water masses of various sampling stations in both rDNA and rRNA derived libraries suggest that this cluster is not an experimental artefact. The recurrence and the overrepresentation in the Perkinsozoa dataset of the cluster A highlight that this cluster play a significant but hitherto unrecognized role in mesopelagic zone that is far to be yet fully explored (Pernice *et al.*, 2015; Edgcomb, 2016). Hence, the major challenge is now to elucidate the ecological role of this cryptic putative parasitic diversity revealed by molecular surveys.

Chapitre 1.2 : References

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Chapitre 1.2 : Supplementary

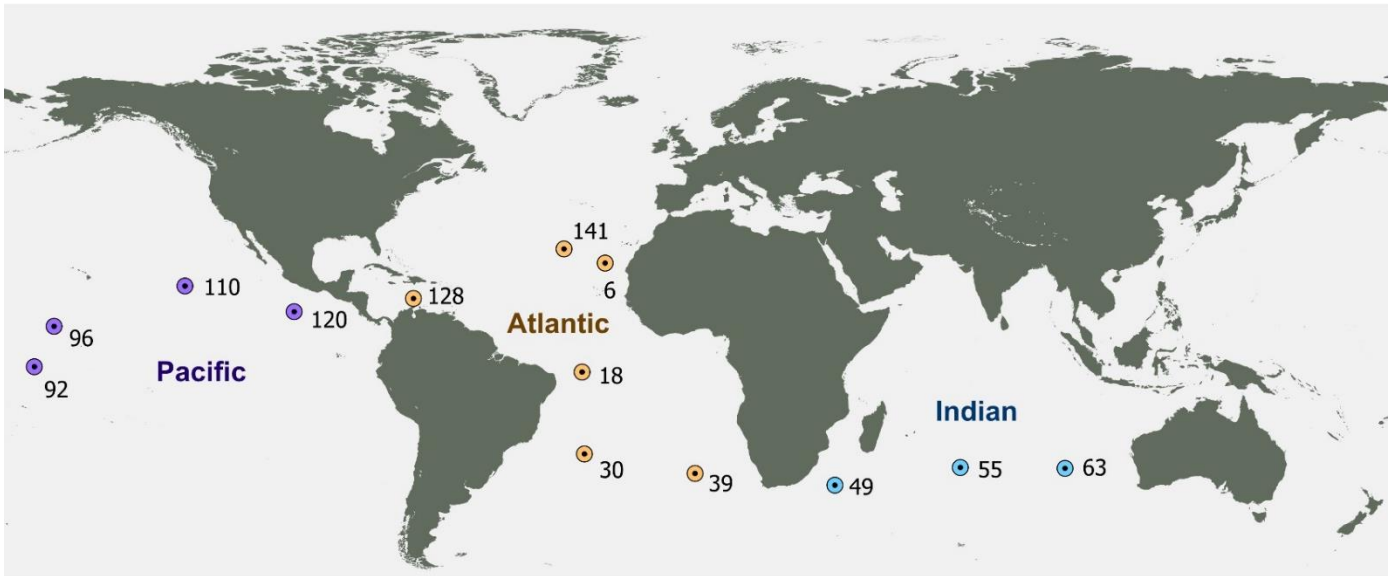
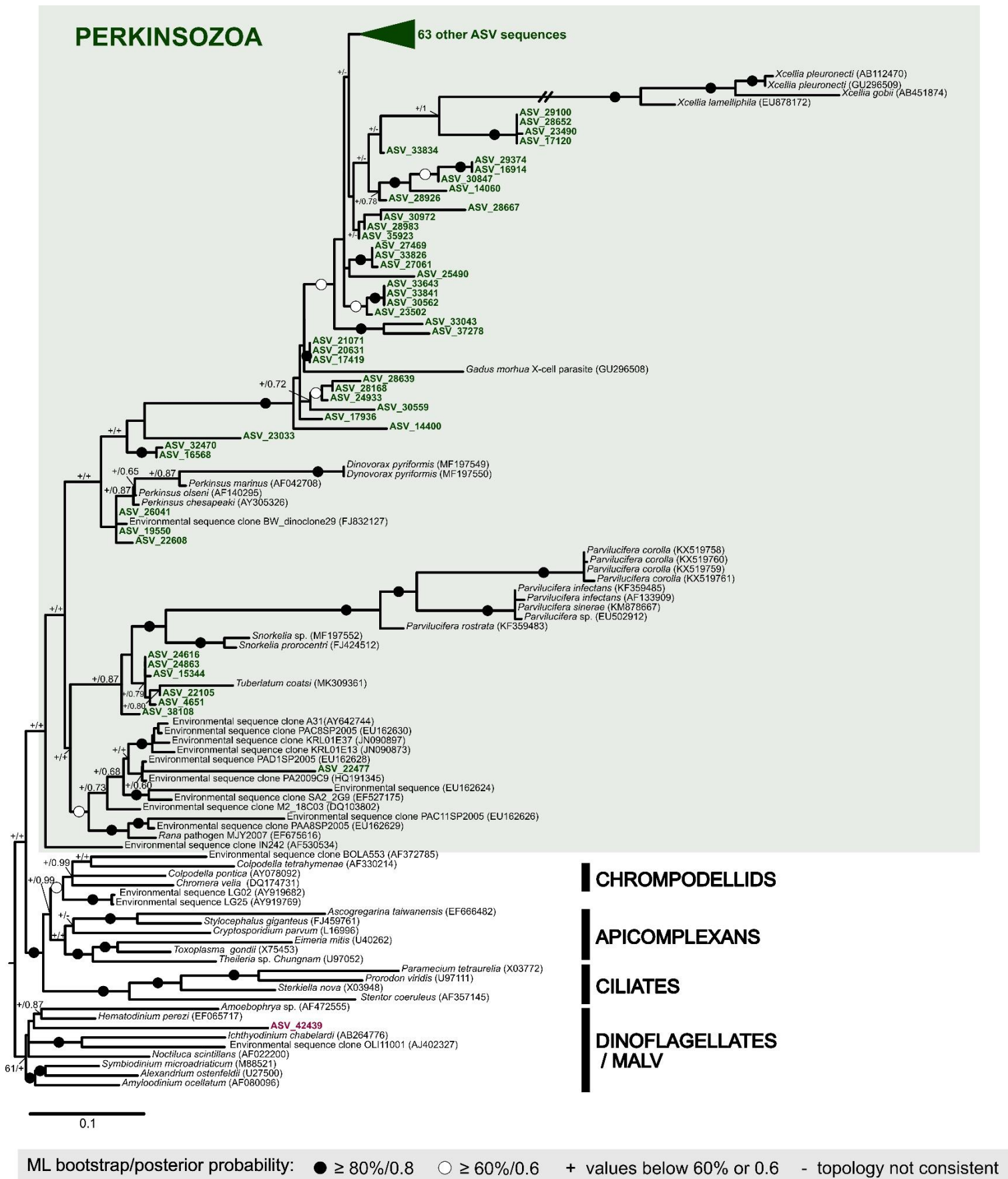


Figure S1. Map of the 13 stations where vertical profiles (from surface to bathypelagic waters) have been sampled during the Circumglobal Malaspina 2010 Expedition. Numbers characterised station name.

Chapitre 1.2 : Diversité environnementale des Perkinsozoa dans les écosystèmes pélagiques



◀ **Figure S2.** Alveolate phylogeny to confirm taxonomic affiliation of Perkinsozoa ASV sequences. RAxML phylogeny of Alveolata 18S rRNA sequences based on the analysis of 60 sequences of 1490 bp and 111 sequences of the V4 region from Malaspina 2010 Circumglobal Expedition sampling. The phylogeny was calculated from 171 taxa and 1489 characters alignment position. ML bootstrap values (1 000 replicates) and Bayesian posterior probability (2 000 000 generations, GTR+G model) were notated using the following convention: support values are summarized by \blacktriangle when $\geq 80\%/0.8$ and white circle when it is not the case but values $\geq 60\%/0.6$. When support value is $<60\%/0.6$ but topology is consistent, the support value is summarized by a '+' and conversely by a '-' if the topology is not consistent. Eight sequences of dinoflagellates, syndiniales and MALV (Marine ALVeolate) were used as an outgroup. Genbank accession numbers were referenced in brackets.

Table S1. Perkinsozoa amplicon single variants (ASVs) from RNA and DNA Malaspina 2010 picoplanktonic dataset: ASV identification, taxonomic assignation and percentage of identity.

ASV code	Supergroup	Group	Species	Species code	Identity (%)
ASV_19550	Alveolata	Perkinsozoa	<i>Perkinsus olsenii</i>	gi 1191448990 gb KX514031.1	93
ASV_22608	Alveolata	Perkinsozoa	<i>Perkinsus olsenii</i>	gi 1191448990 gb KX514031.1	92
ASV_23033	Alveolata	Perkinsozoa	<i>Perkinsus olsenii</i>	gi 1191448990 gb KX514031.1	82
ASV_26041	Alveolata	Perkinsozoa	<i>Perkinsus olsenii</i>	gi 1191448990 gb KX514031.1	93
ASV_16568	Alveolata	Perkinsozoa	<i>Perkinsus qugwadi</i>	gi 441415474 dbj AB716689.1	89
ASV_32470	Alveolata	Perkinsozoa	<i>Perkinsus qugwadi</i>	gi 441415474 dbj AB716689.1	90
ASV_4651	Alveolata	Perkinsozoa	<i>Dinovorax pyriformis</i>	gi 1248939539 gb MF197550.1	100
ASV_15344	Alveolata	Perkinsozoa	<i>Dinovorax pyriformis</i>	gi 1248939539 gb MF197550.1	96
ASV_22105	Alveolata	Perkinsozoa	<i>Dinovorax pyriformis</i>	gi 1248939539 gb MF197550.1	95
ASV_24616	Alveolata	Perkinsozoa	<i>Dinovorax pyriformis</i>	gi 1248939539 gb MF197550.1	95
ASV_24863	Alveolata	Perkinsozoa	<i>Dinovorax pyriformis</i>	gi 1248939539 gb MF197550.1	96
ASV_27057	Alveolata	Perkinsozoa	<i>Dinovorax pyriformis</i>	gi 1248939539 gb MF197550.1	95
ASV_32262	Alveolata	Perkinsozoa	<i>Dinovorax pyriformis</i>	gi 1248939539 gb MF197550.1	94
ASV_38108	Alveolata	Perkinsozoa	<i>Dinovorax pyriformis</i>	gi 1248939539 gb MF197550.1	96
ASV_14400	Alveolata	Perkinsozoa	X-cell	gi 1148361579 gb KY628810.1	84
ASV_17120	Alveolata	Perkinsozoa	X-cell	gi 1148361579 gb KY628810.1	82
ASV_17419	Alveolata	Perkinsozoa	X-cell	gi 1148361579 gb KY628810.1	88
ASV_17936	Alveolata	Perkinsozoa	X-cell	gi 1148361579 gb KY628810.1	90
ASV_20631	Alveolata	Perkinsozoa	X-cell	gi 1148361579 gb KY628810.1	87
ASV_21071	Alveolata	Perkinsozoa	X-cell	gi 1148361579 gb KY628810.1	87
ASV_23490	Alveolata	Perkinsozoa	X-cell	gi 1148361579 gb KY628810.1	82
ASV_24933	Alveolata	Perkinsozoa	X-cell	gi 1148361579 gb KY628810.1	87
ASV_28168	Alveolata	Perkinsozoa	X-cell	gi 1148361579 gb KY628810.1	88
ASV_28639	Alveolata	Perkinsozoa	X-cell	gi 1148361579 gb KY628810.1	86
ASV_28652	Alveolata	Perkinsozoa	X-cell	gi 1148361579 gb KY628810.1	82
ASV_29100	Alveolata	Perkinsozoa	X-cell	gi 1148361579 gb KY628810.1	82
ASV_30559	Alveolata	Perkinsozoa	X-cell	gi 1148361579 gb KY628810.1	86
ASV_13538	Alveolata	X-Cell	NA	NA	NA
ASV_14060	Alveolata	X-Cell	NA	NA	NA
ASV_14675	Alveolata	X-Cell	NA	NA	NA
ASV_15583	Alveolata	X-Cell	NA	NA	NA
ASV_16233	Alveolata	X-Cell	NA	NA	NA
ASV_16914	Alveolata	X-Cell	NA	NA	NA
ASV_17003	Alveolata	X-Cell	NA	NA	NA
ASV_17318	Alveolata	X-Cell	NA	NA	NA
ASV_18816	Alveolata	X-Cell	NA	NA	NA
ASV_19156	Alveolata	X-Cell	NA	NA	NA
ASV_21285	Alveolata	X-Cell	NA	NA	NA
ASV_21607	Alveolata	X-Cell	NA	NA	NA
ASV_21791	Alveolata	X-Cell	NA	NA	NA
ASV_22325	Alveolata	X-Cell	NA	NA	NA
ASV_22697	Alveolata	X-Cell	NA	NA	NA
ASV_23032	Alveolata	X-Cell	NA	NA	NA
ASV_23061	Alveolata	X-Cell	NA	NA	NA
ASV_23089	Alveolata	X-Cell	NA	NA	NA
ASV_23488	Alveolata	X-Cell	NA	NA	NA
ASV_23502	Alveolata	X-Cell	NA	NA	NA
ASV_24187	Alveolata	X-Cell	NA	NA	NA
ASV_25440	Alveolata	X-Cell	NA	NA	NA
ASV_25490	Alveolata	X-Cell	NA	NA	NA
ASV_25758	Alveolata	X-Cell	NA	NA	NA
ASV_26043	Alveolata	X-Cell	NA	NA	NA
ASV_26111	Alveolata	X-Cell	NA	NA	NA
ASV_26367	Alveolata	X-Cell	NA	NA	NA
ASV_26436	Alveolata	X-Cell	NA	NA	NA
ASV_26695	Alveolata	X-Cell	NA	NA	NA
ASV_26756	Alveolata	X-Cell	NA	NA	NA

Chapitre 1.2 : Diversité environnementale des Perkinsozoa dans les écosystèmes pélagiques

ASV_27061	Alveolata	X-Cell	NA	NA	NA
ASV_27113	Alveolata	X-Cell	NA	NA	NA
ASV_27469	Alveolata	X-Cell	NA	NA	NA
ASV_28667	Alveolata	X-Cell	NA	NA	NA
ASV_28926	Alveolata	X-Cell	NA	NA	NA
ASV_28983	Alveolata	X-Cell	NA	NA	NA
ASV_29076	Alveolata	X-Cell	NA	NA	NA
ASV_29374	Alveolata	X-Cell	NA	NA	NA
ASV_29476	Alveolata	X-Cell	NA	NA	NA
ASV_29530	Alveolata	X-Cell	NA	NA	NA
ASV_29576	Alveolata	X-Cell	NA	NA	NA
ASV_29940	Alveolata	X-Cell	NA	NA	NA
ASV_30528	Alveolata	X-Cell	NA	NA	NA
ASV_30536	Alveolata	X-Cell	NA	NA	NA
ASV_30546	Alveolata	X-Cell	NA	NA	NA
ASV_30562	Alveolata	X-Cell	NA	NA	NA
ASV_30847	Alveolata	X-Cell	NA	NA	NA
ASV_30972	Alveolata	X-Cell	NA	NA	NA
ASV_31635	Alveolata	X-Cell	NA	NA	NA
ASV_31707	Alveolata	X-Cell	NA	NA	NA
ASV_31725	Alveolata	X-Cell	NA	NA	NA
ASV_31733	Alveolata	X-Cell	NA	NA	NA
ASV_32384	Alveolata	X-Cell	NA	NA	NA
ASV_32401	Alveolata	X-Cell	NA	NA	NA
ASV_33043	Alveolata	X-Cell	NA	NA	NA
ASV_33586	Alveolata	X-Cell	NA	NA	NA
ASV_33643	Alveolata	X-Cell	NA	NA	NA
ASV_33680	Alveolata	X-Cell	NA	NA	NA
ASV_33826	Alveolata	X-Cell	NA	NA	NA
ASV_33829	Alveolata	X-Cell	NA	NA	NA
ASV_33834	Alveolata	X-Cell	NA	NA	NA
ASV_33835	Alveolata	X-Cell	NA	NA	NA
ASV_33841	Alveolata	X-Cell	NA	NA	NA
ASV_33884	Alveolata	X-Cell	NA	NA	NA
ASV_34569	Alveolata	X-Cell	NA	NA	NA
ASV_34574	Alveolata	X-Cell	NA	NA	NA
ASV_34662	Alveolata	X-Cell	NA	NA	NA
ASV_35311	Alveolata	X-Cell	NA	NA	NA
ASV_35923	Alveolata	X-Cell	NA	NA	NA
ASV_36317	Alveolata	X-Cell	NA	NA	NA
ASV_36940	Alveolata	X-Cell	NA	NA	NA
ASV_37014	Alveolata	X-Cell	NA	NA	NA
ASV_37023	Alveolata	X-Cell	NA	NA	NA
ASV_37144	Alveolata	X-Cell	NA	NA	NA
ASV_37278	Alveolata	X-Cell	NA	NA	NA
ASV_37900	Alveolata	X-Cell	NA	NA	NA
ASV_38067	Alveolata	X-Cell	NA	NA	NA
ASV_38254	Alveolata	X-Cell	NA	NA	NA
ASV_38281	Alveolata	X-Cell	NA	NA	NA
ASV_42165	Alveolata	X-Cell	NA	NA	NA
ASV_42203	Alveolata	X-Cell	NA	NA	NA
ASV_42524	Alveolata	X-Cell	NA	NA	NA
ASV_42439	Alveolata	X-Cell	NA	NA	NA

PARTIE 2

Cas d'étude : Détection et distribution de la co-infection entre deux espèces de *Perkinsus* chez la palourde japonaise, *R. philippinarum*, à l'échelle populationnelle.

Chapitre 2.1 : Développement d'une méthode moléculaire basée sur la PCR quantitative en temps réel TaqMan pour la détection simultanée de *Perkinsus olseni* et *P. chesapeakei* chez les palourdes japonaises, *Ruditapes philippinarum*, du bassin d'Arcachon.

Avant-propos

Dans cette **deuxième partie** de thèse, nous nous sommes intéressés à un cas particulier d'infection par deux espèces de *Perkinsus* (Alveolata), *P. olseni* et *P. chesapeakei*, au sein d'une population d'hôte de palourdes japonaises, *R. philippinarum*, dans le bassin d'Arcachon. Aujourd'hui, l'agent étiologique *Perkinsus olseni* est largement reconnu comme une menace majeure pour le secteur conchylicole à travers le monde. Pour la première fois en France, Arzul *et al.* (2014) ont détecté une autre espèce, *Perkinsus chesapeakei*, co-infectant sporadiquement les populations de palourdes européennes, *R. decussatus*, et japonaises, *R. philippinarum*, en association avec *P. olseni*. La résolution des infections cryptiques potentielles affectant *Ruditapes philippinarum* est essentielle pour adapter les stratégies de gestion des ressources conchylicoles. Ainsi, au cours de ce **Chapitre 2.1**, nous avons développé une méthode moléculaire basée sur la PCR quantitative en duplex en temps réel pour permettre la détection simultanée de ces deux parasites, *P. olseni* et *P. chesapeakei*, dans les différents tissus de palourdes : branchies, glande digestive, pied, manteau, muscle adducteur et le reste du corps mou. Le bassin d'Arcachon est un écosystème modèle qui a été choisi pour l'étude de la Perkinsose durant cette thèse, car la prévalence de *Perkinsus* spp. y est forte (>70 %), régulièrement suivi et qu'elle ne cause pas de mortalités saisonnières comme déjà cela a déjà été observé en Espagne ou au Portugal. Dans l'ordre chronologique, nous avons réalisé en 2017 un premier échantillonnage de palourdes japonaises dans le bassin d'Arcachon qui nous a permis de découvrir l'existence de *P. olseni* et *P. chesapeakei* et de les mettre en culture. L'occurrence de *P. chesapeakei* était surprenante car cette espèce n'avait encore jamais été détectée dans cette zone malgré les nombreux suivis. A ce stade aucune donnée n'était disponible sur la prévalence de ces co-infections, sur leur conséquence sur l'hôte ou encore sur leur dynamique d'infection. Ainsi, nous avons décidé de mettre en place et d'optimiser une méthode moléculaire de qPCR en duplex afin de quantifier simultanément ces deux parasites

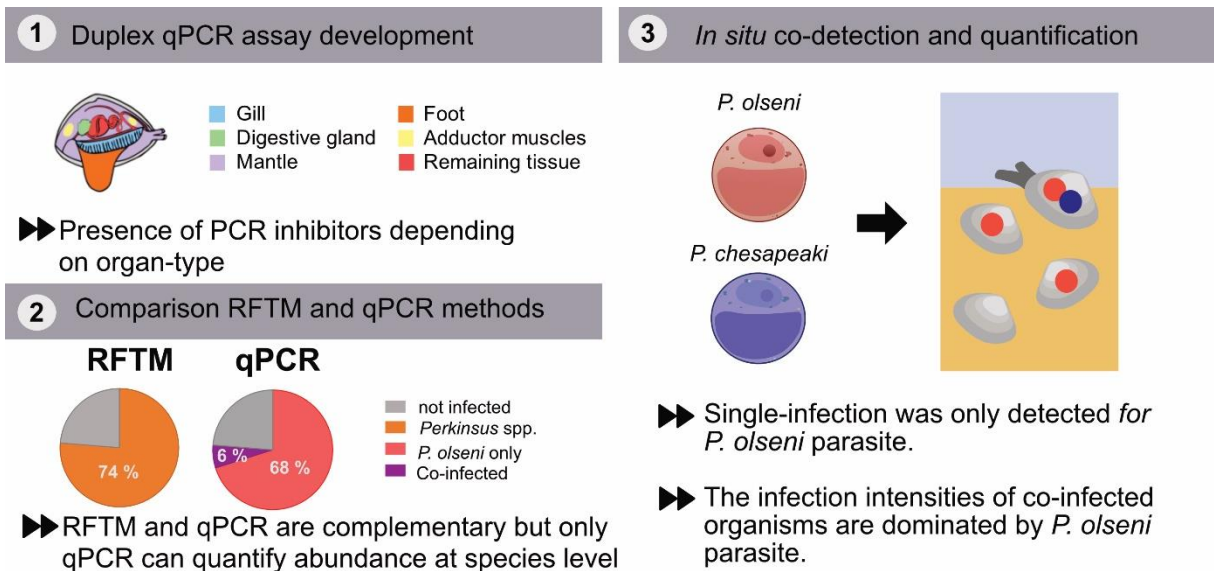
Chapitre 2.1 : Développement de la qPCR en duplex pour la détection de co-infection

dans des échantillons *in situ*. Tous les organes du corps de la palourde ont été considérés dans l'hypothèse où la présence des deux parasites puisse être localisée à l'intérieur de l'hôte. Pour cela, nous avons donc vérifié la présence d'éventuels inhibiteurs de la PCR dans des échantillons de tissus de l'hôte. Ensuite, en utilisant les branchies comme modèle, l'adéquation de la technique moléculaire a été vérifiée par rapport à la méthodologie du milieu liquide de thioglycollate de Ray (RFTM), recommandée par l'Organisation mondiale de la santé animale (O.I.E). Ce **Chapitre 2.1** a pour but le développement méthodologique d'un outil moléculaire pour comprendre la co-infection et sa distribution dans les populations de japonaises.

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.

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Abstract

The aetiological agent *Perkinsus olseni* is globally recognised as a major threat for shellfish production considering its large geographical distribution across Asia, Europe, Australia and South America. Another species, *Perkinsus chesapeaki*, which had never been officially associated with mortality events, was recently detected along the French coasts infecting clam populations sporadically in association with *P. olseni*. Disclosing potential cryptic infections affecting *Ruditapes philippinarum* is essential to adapt the host resource management strategy. 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

Chapitre 2.1 : Développement de la qPCR en duplex pour la détection de co-infection

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 $2 \text{ ng} \cdot \mu\text{L}^{-1}$, the adductor muscle and the foot have an intermediate inhibition of $5 \text{ ng} \cdot \mu\text{L}^{-1}$ and the gills and digestive gland do not show any inhibition of the qPCR reaction even at the highest host gDNA concentration of $20 \text{ ng} \cdot \mu\text{L}^{-1}$. Then, using the gills as a template, the suitability of the molecular technique was checked in comparison with the Ray's Fluid Thioglycollate Medium methodology recommended by the World Organisation for Animal Health. The duplex qPCR method brought new insights and unveil cryptic infections as the co-occurrence of *P. olseni* and *P. chesapeakei* 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 the global bivalve production (F.A.O, 2018). Introduced in Europe in the 1970s (Flassch and Leborgne, 1994) for aquaculture purposes, this bivalve is currently the third most important bivalve production 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 as bacteria, virus, or 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 un-precedent environmental transformation and the survey of these epizooties 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 recurrent epidemic disease that occurred worldwide in commercially important marine molluscs including oysters, clams and abalones. Two main parasites, *Perkinsus marinus* and *Perkinsus olseni*, identified as the infectious agent of Perkinsosis, are classified as notifiable pathogen in the Organisation for Animal Health (O.I.E)-list diseases since 2006 (O.I.E, 2019a). *P. marinus* is responsible of 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 related to clams 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 to abalone mortality in Australia (Goggin and Lester, 1995). However, infection by *Perkinsus* species does not drive inexorably to the host death but can induce sublethal effects resulting in growth delay, reproductive activity reduction, deleterious effects on filtering activity and digestion leading to a global decrease in host fitness 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 help unravelling 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 sample have been reported (e.g. Reece *et al.*, 2008, Coss *et al.*, 2001, Takahashi *et al.*, 2009). In Europe, two *Perkinsus* species, *P. olseni* and *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). Conversely to *P. olseni*, *P. chesapeaki* has never been officially associated with a mass mortality event despite some suspicions about 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 the 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 result of a process reality where interaction network includes 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 thioglycollate 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; O.I.E, 2019b; Ray, 1952). The RFTM methodology, which is easy to do, 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 O.I.E, is very useful to visualise lesions and parasite distribution in tissues even if it is time-consuming and less sensitive (O.I.E, 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 the disease outcome.

The qPCR methodology is an accurate and sensitive tool which should be more considered 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 *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 (Itoiz *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 km² where intertidal mudflat macrofauna is mainly dominated by *R. philippinarum* which could represent up to 90% of the total biomass (Bertignac *et al.*, 2001).

Chapitre 2.1 : Développement de la qPCR en duplex pour la détection de co-infection

Of the 55 clams sampled, fifty were dissected for RFTM and molecular assays. Briefly, clams were opened on ice and one gill was weighted and incubated in RFTM supplemented with antibiotics (penicillin $66 \mu\text{g.mL}^{-1}$ and streptomycin $32 \mu\text{g.mL}^{-1}$) and antimycotic (nystatin 0.04 mg.mL^{-1}) to process counting method according to Choi *et al.* (1989). The rest 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 weighted and preserved separately in ethanol 80 % at 4°C until further molecular analysis. Shells were measured and weighted. Five clams were kept in a 40L tank of filtered ($0.2 \mu\text{m}$) sea water at 15°C for 10 days prior using them for establishing *P. olseni* and *P. chesapeaki* cultures.

2.2. Ray's fluid thioglycollate medium (RFTM)

Body-burden assays were proceeded following the RFTM method as recommended by the O.I.E. (Ray, 1952; Choi *et al.*, 1989; O.I.E, 2019b). Briefly, after incubation in RFTM in the dark for five days at room temperature, allowing the enlargement of trophozoites (= hypnospores formation) (Ray, 1952), the gill tissue samples were digested with 2M NaOH solution for 3 h at 60°C preserving the hypnospores 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 culture following a simplified protocol from Casas *et al.* (2002a). In short, gill tissues samples were excised and decontaminated with five successive baths of antibiotics 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 waiting for trophozoites proliferation. Five cultures were initiated from the five gills 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 culture of *P. olseni* and *P. chesapeaki* were diluted at $2\ 000\ \text{cell.mL}^{-1}$ 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 first visible colonies. Each colony was checked using 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 beads size (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). Tissues were grinded and homogenized following two bead beating cycles (45 seconds of bead beating at $6\ \text{m. s}^{-1}$ following by 20 seconds stop) into cooling rack of the FastPrep-24 5G (MP Biomedicals). After this step, β -mercaptoethanol (0.2 %) and proteinase K ($0.1\ \text{mg.mL}^{-1}$) were then added in each tube and samples were incubated 30 min at 60°C. Foot tissue samples were exclusively incubated 12 hours due to its extremely hard muscled structure. Lysates were mixed in chloroform/isoamylalcohol (24:1, v/v) and emulsified prior 10 min spin at 18.000 g rpm in a cooled microcentrifuge (4°C). This step was repeated twice for foot tissue samples. Aqueous phases were treated with RNase solution ($10\ \text{mg.mL}^{-1}$) (Sigma-Aldrich) for 30 minutes at 37 °C prior DNA precipitation with cold 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 1 % agarose gel, 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. The 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 of *P. olseni* monoclonal culture and MW187112 of *P. chesapeaki* monoclonal culture).

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

Plasmids standards are an easy and accurate method for relative quantification of parasite in host tissue, DNA extraction protocol and degree of trophozoites maturation (e.g. schyzogony). The methodology used to establish the two standards is based on the duplex study between *Perkinsus* and *Haplosporidium* species from Xie *et al.* (2013). The ITS sequences were cloned in pGEM-T easy vector system I (Promega) and transformed into homemade competent *Escherichia coli* XGold following the manufacturer recommendations. Clones were blue/white screened and numerous clones per library were selected for overnight growing in Lysogeny Broth media (LB) at 37°C. Plasmids DNA were 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: $molecule/\mu l = a / (3\ 665\ bp \times 660\ Da) \times$

6.022×10^{23} where a is the DNA plasmid concentration, 3665 bp is the full-length plasmid including the vector (3 015 bp) and the amplified fragment (650 bp), 660 Da is the molecular weight of one bp and 6.022×10^{23} is the molecular Avogadro constant.

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

Showing a high genetic polymorphism suitable for species-level discrimination, the ITS regions have been commonly selected as good candidate to develop specific primers (Casas *et al.*, 2002a; b; O.I.E, 2019b). Primers and probes were designed using Primer3Plus (qPCR settings) (Untergasser *et al.*, 2012) with template sequences: JQ669642, KX514117, KX514123, LC431768, DQ516714, MG733367, MG733365, KP764683, KX514103, KP764681, FJ481986, EU293848 for *P. olseni* and EU919479, EU919489, EU919501, AY876312, AY876314, EU919465, EU919496, AY876318, AY876316, MF186901, MF186913, MF186910 for *P. chesapeaki*. To optimise the duplex real-time PCR, set of primers and probes were designed with close fusion temperature (T_m) and close amplicon length (Figure 1; Table 1). Specificity were checked *in silico* using: (1) the ARB Software (Ludwig *et al.*, 2004) against a multiple sequence alignment of *Perkinsus* ITS1- 5.8S – ITS2 region made from sequences available on the National Center for Biotechnology Information (NCBI) (accessed March 2019) (Table S2); (2) Primer-BLAST (accessed March 2019) against NCBI non redundant (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.gov) (Table S4). Secondary structure and dimers formation were checked using the OligoEvaluator (Sigma-Aldrich).

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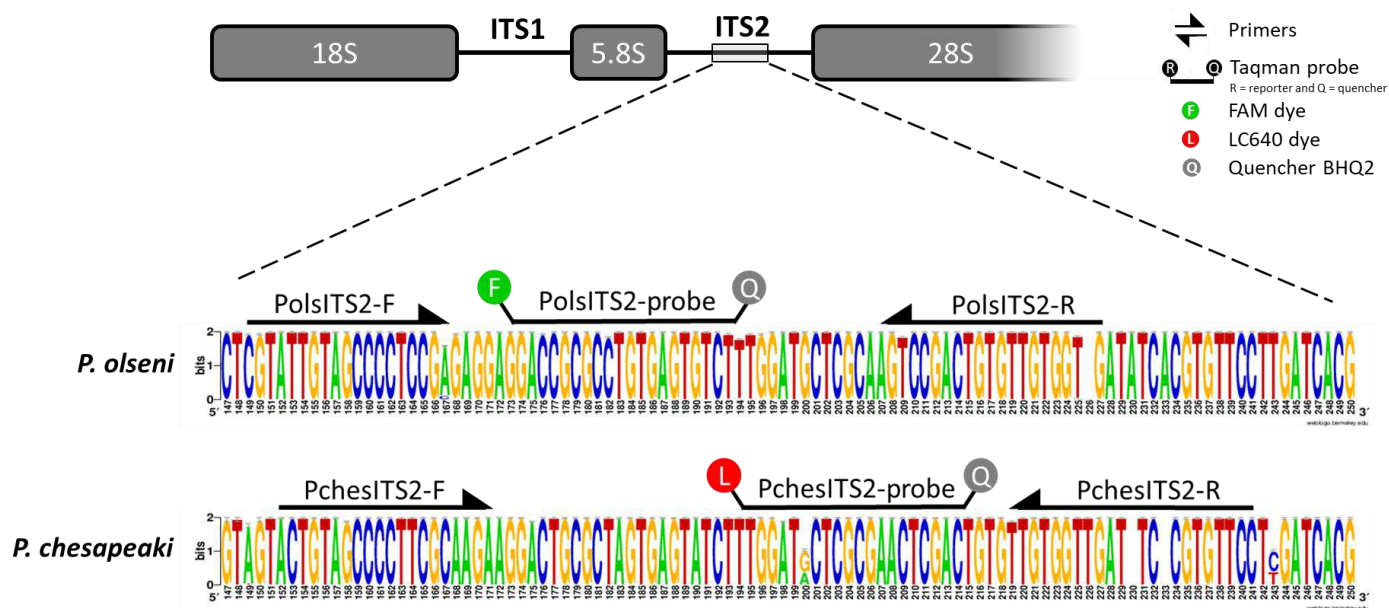


Figure 1. Schematic position of primers and probes designed for duplex TaqMan PCR within the ITS1-5.8S-ITS2 sequence region of ribosomal genes of *Perkinsus* spp.

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 <i>P. olseni</i>	PolITS2-F	CACCACAACACAGTCGGAC	58,8	
	PolITS2-R	CGTATTGTAGCCCCCTCCGA	58,8	76 bp
	PolITS2-probe	FAM -GACTCACAGGCGGTCC-[BHQ2]	65,7	
ITS 2 <i>P. chesapeaki</i>	PchesITS2-F	GGAACACGGAATCAACCACA	57,3	
	PchesITS2-R	ACTGTAGCCCCCTTCGCAAG	58,8	87 bp
	PchesITS2-probe	LC640 -AGTCGAGTTCGCGAGCATCCAA-[BHQ2]	63,3	

2.5.4. Real-time PCR assays development

Real-time PCR 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*. Gills 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 μ M and 2 μ M 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 MgCl₂. The reaction volume for duplex qPCR consisted of 1X LightCycler 480 Probes Master premixed, 0.2 μ M of primer PolsITS2-F, 0.2 μ M of PolsITS2-R, 0.2 μ M of PchesITS2-F, 0.2 μ M of PchesITS2-R, 0.5 μ M of PolsITS2-probe (FAM), 0.5 μ M of PchesITS2-probe (LC640), and 5 μ L of DNA sample with adjusted concentration. The reaction volume for simplex qPCR consisted of 1X LightCycler 480 Probes Master premixed, 0.2 μ M of primer PolsITS2-F or PchesITS2-F, 0.2 μ M of PolsITS2-R or PchesITS2-R, 0.5 μ M of PolsITS2-probe or PchesITS2-probe, 5 μ L of DNA sample with adjusted concentration. The thermal cycling conditions were as followed: 5 min of pre-incubation at 95 °C followed by 45 cycles of amplification containing 10 s of denaturation step at 95°C and 20 s of annealing step 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). PCR assay finally terminated by a last cooling period of 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 plasmids standards of *P. olsenii* 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 (2.5×10^1 to 2.5×10^6 total copies) of the reference standards. For each dilution, the mean of Ct values and standard-deviation 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 in duplex qPCR assay to establish reference values of non-inhibited reaction for both species. The sensitivity of the duplex qPCR was estimated on ITS1-5.8S-ITS2 of *P. olsenii* and *P. chesapeaki* plasmids with 10-fold serial dilution from 2.5×10^1 to 2.5×10^6 total copies in triplicates. The 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% represent an optimal doubling amount of DNA and; 2) the limit of detection (LOD), which is the last 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, 3 *Perkinsus*-free clams were selected to determine the possible inhibitory effect for each organ type. To assess a global trend of the 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. olsenii* and *P. chesapeaki* plasmids DNA (2.5×10^1 to 2.5×10^6 total copies) supplemented with a dilution

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(2, 5, 10, and 20 ng.µL⁻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 extract of gDNA organs 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 the 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 culture method and the duplex real-time PCR method, all 50 gill DNA samples were tested in duplex qPCR. To ensure a reliable comparison between infected individuals, the concentration of all gill DNA samples were adjusted at 20 ng.µL⁻¹ as determined above. The qPCR was then run in triplicates using the same parameters as above. The 10-fold dilution plasmids standards for *P. olseni* and *P. chesapeaki* were added to the run to determine copies concentration 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 extract at several concentrations (20, 10, 5, 2 ng.µL⁻¹) were compared to determine which concentration represents 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 large majority of variables tested for each inhibitory condition fulfilled conditions of applications for ANCOVA. Plasmids concentrations were log-transformed. Regression model was fitting (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 the organ type on Ct values depending on organ concentration (covariate). Curves showing no significant difference with the standard 10-fold plasmids dilution were selected as good candidates for *Perkinsus* detection. Absence of difference from the standard demonstrated a non-inhibited reaction of amplification by the 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 paired of positive samples and paired 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 in RFTM and negative in qPCR assay, and vice versa), is the percentage of 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 nb. of copies.g of wet gill⁻¹; x : log of RFTM-infection intensity in nb. of cells.g of wet gill⁻¹).

3. Results

3.1. Specificity of the PCR assay

Primers and probes design is a crucial step for an accurate duplexing real-time PCR assay. *In silico* tests demonstrated that primers and probes did not match with 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 *P. olseni* primers (PolsITS2_F/PolsITS2_R) and probe (PolsITS2_probe) set, a significant amplification (Ct: 26.4 ± 0.07) is detected using *P. olseni* gDNA as template with the filter FAM (465-510 wavelengths) corresponding dye excitation wavelength coupled with *P. olseni* probe (Figure 2). As expected, no amplification is 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 amplifies (Ct: 26.7 ± 0.09) only with *P. chesapeaki* gDNA with the suitable filter LC640 (618-660 wavelengths) and no amplification is detected, for other wavelengths or other templates as *P. olseni* gDNA, host gDNA and pure water (Figure 2). These results demonstrate that the qPCR primers and probes sets are respectively specific to the targeted gDNA and without any other cross detection.

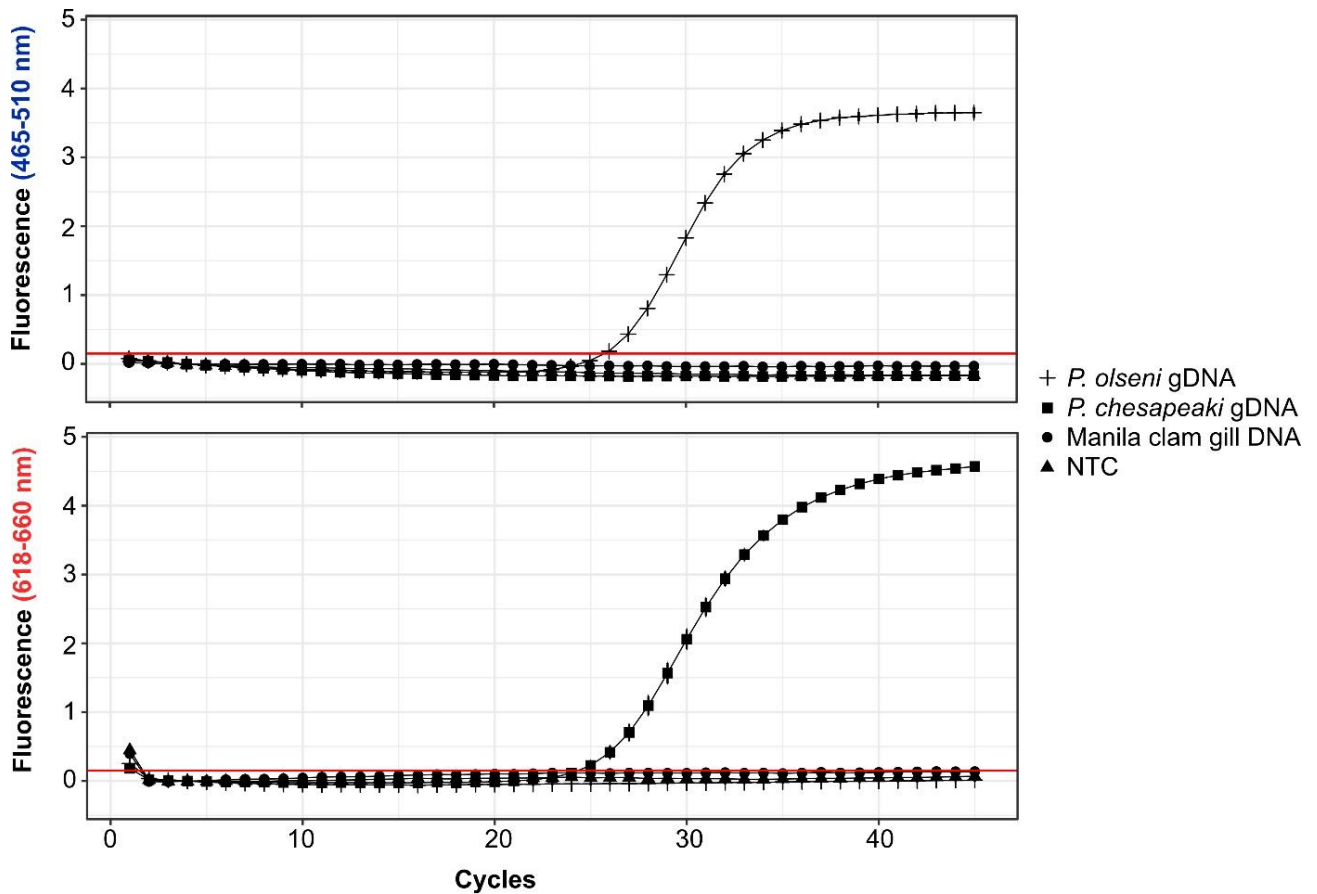


Figure 2. Specificity of primers and probes designed for the duplex qPCR assay. Primers and probes specificity were tested by conducting duplex real-time PCR on *P. olsenii* gDNA (1 pg), *P. chesapeaki* gDNA (1 pg), *R. philippinarum* gills gDNA (50 ng) and pure water. Positive amplification is significant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

3.2. Standards PCR sensitivity and repeatability

Regarding the unknown number of ribosomal operon and the ploidy level of each life stage, plasmids offer a good opportunity to overcome this limit using a universal reproducible and stable standard of qPCR assays (Dhanasekaran *et al.*, 2010). The ITS sequence of each parasitic culture was isolated via plasmids purification from cloned and transformed competent bacteria. Two standard curves (*P. olsenii*: $y = -3.33x + 39.49$; *P. chesapeaki*: $y = -3.38x + 40.29$; Figure 3) were generated from 10-fold serial dilution from 2.5×10^1 to 2.5×10^6 copies of ITS rDNA plasmid. Ct values of both standard curves show a strong linear correlation with the targeted template (*P. olsenii*: $R^2 = 0.95$; *P. chesapeaki*: $R^2 = 0.95$; Figure 3). The limit of detection is set

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at 25 copies of the target DNA in 10 μL reaction volume; below this number of plasmid copies (2.5 copies; data not shown), triplicates expose non-replicable Ct values. DNA amplification is 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 is estimated for *P. olseni* standard between 3 to 8 % (Table 2).

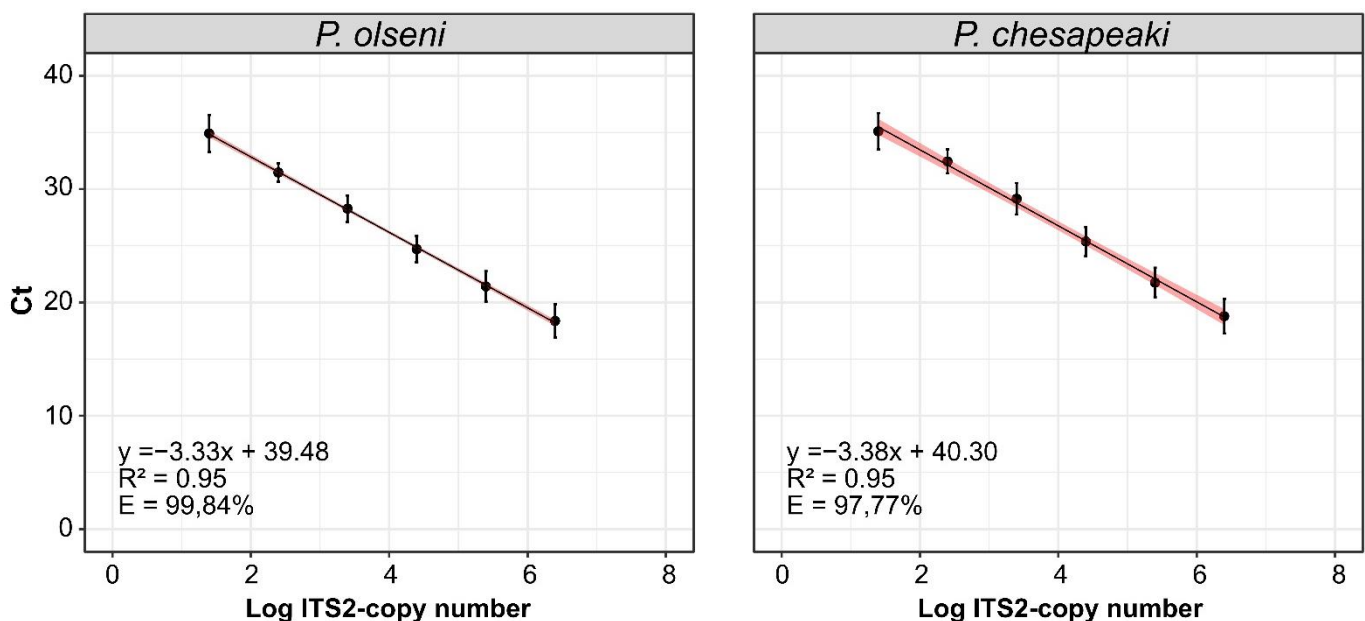


Figure 3. Standards and efficiency evaluation of real-time PCR assays. Standard curves based on *P. olseni*-plasmids and on *P. chesapeaki*-plasmids serial dilution were determined for downstream analysis. *P. olseni* standard relationship: $y = -3.33x + 39.48$; *P. chesapeaki* standard relationship: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

Table 2. Repeatability of the duplex qPCR assay estimated by the coefficient of variation (CV; %) for *P. olseni* and *P. chesapeakei* 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
<i>P. olseni</i> star	5%	3%	4%	5%	6%	8%
<i>P. chesapeak</i>	5%	3%	5%	5%	6%	8%

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

Organs 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 $\mu\text{g}\cdot\mu\text{L}^{-1}$). Ct values vary from *P. olseni* and *P. chesapeakei* standards depending on organ type and gDNA concentration used for the amplification (Figure 4). Results of ANCOVA and efficiencies are synthesised in the 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*-plasmids detection

P. olseni-standards containing only plasmids represent 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 do not influence the linear 10-fold dilution curves (ANCOVA, p-value >0.05) (Figure 4A). With no difference observed with the standard curve, the maximum concentration is 20 $\text{ng}\cdot\mu\text{L}^{-1}$ for gDNA of both gills (efficiency (E) = 100.3 %) and digestive gland (E = 100.4 %). For the adductor muscle, solely the amplification curve corresponding to 20 $\text{ng}\cdot\mu\text{L}^{-1}$ shows significant difference with the *P. olseni* standard (ANCOVA, F=5.53, p-value < 0.05) and has a high efficiency of 115.4 %. Maximum concentrations showing no difference with the standard are the 10 $\text{ng}\cdot\mu\text{L}^{-1}$ and 5 $\text{ng}\cdot\mu\text{L}^{-1}$ curves (ANCOVA, p-value >0.05). The 5 $\text{ng}\cdot\mu\text{L}^{-1}$ curve is preferred for its optimal efficiency of 98,7 %. For the foot, the amplification curves corresponding to 10 $\text{ng}\cdot\mu\text{L}^{-1}$ and 20 $\text{ng}\cdot\mu\text{L}^{-1}$ show significant differences with the *P. olseni* standard (ANCOVA, $F_{10\text{ng}/\mu\text{L}}=8.4$, p-value $_{10\text{ng}/\mu\text{L}} < 0.01$; $F_{20\text{ng}/\mu\text{L}}=17.6$, p-value $_{20\text{ng}/\mu\text{L}} < 0.001$). The maximum concentration with no difference with the standard is the 5 $\text{ng}\cdot\mu\text{L}^{-1}$ curve (E = 92.8 % ; ANCOVA, p-value >0,05). For the mantle,

the $2 \text{ ng} \cdot \mu\text{L}^{-1}$ is the maximum concentration that does not alter the linear 10-fold dilution curve ($E = 92 \%$; ANCOVA, $p\text{-value} > 0.05$). Even if the amplification curves for $5 \text{ ng} \cdot \mu\text{L}^{-1}$ and $20 \text{ ng} \cdot \mu\text{L}^{-1}$ show non-statistical difference with the standard, their shifts are out of the error range which highlight an important variability for these concentrations (Figure 4A). For the remaining tissue, the $2 \text{ ng} \cdot \mu\text{L}^{-1}$ is the maximum concentration of gDNA that does not alter the linear 10-fold dilution curve ($E = 105.6 \%$; ANCOVA, $p\text{-value} > 0.05$). Other concentrations show significant differences or too high efficiencies (e.g. the $10 \text{ ng} \cdot \mu\text{L}^{-1}$ curve : $E = 121.7 \%$) to be selected.

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

Same trends are observed for *P. chesapeaki* where standard containing only plasmids represents the positive control without any inhibitory effects (Figure 3). For gill and digestive gland, the addition of gDNA extract does not influence the linear 10-fold dilution curves at none of the tested concentration (ANCOVA, $p\text{-value} > 0.05$) (Figure 4B). With no difference observed with the standard curve, the maximum concentration is $20 \text{ ng} \cdot \mu\text{L}^{-1}$ for gDNA of both gills ($E = 98.3 \%$) and digestive gland ($E = 97.2 \%$). For the adductor muscle, the curve corresponding to $10 \text{ ng} \cdot \mu\text{L}^{-1}$ shows significant difference with the standard (ANCOVA, $F_{10\text{ng}/\mu\text{L}}=5.5$, $p\text{-value} < 0.05$). The maximum concentration showing no differences with the standard is the $5 \text{ ng} \cdot \mu\text{L}^{-1}$ curve ($E = 96.3 \%$; ANCOVA, $p\text{-value} > 0.05$). For the foot, the gDNA concentrations related to $10 \text{ ng} \cdot \mu\text{L}^{-1}$ and $20 \text{ ng} \cdot \mu\text{L}^{-1}$ show significant differences with the *P. chesapeaki* standard (ANCOVA, $F_{10\text{ng}/\mu\text{L}}= 26.6$, $p\text{-value}_{10\text{ng}/\mu\text{L}} < 0.001$; $F_{20\text{ng}/\mu\text{L}}= 16.1$, $p\text{-value}_{20\text{ng}/\mu\text{L}} < 0.001$). The maximum concentration of foot gDNA extract with no difference with the standard is the $5 \text{ ng} \cdot \mu\text{L}^{-1}$ curve ($E = 89.3 \%$; ANCOVA, $p\text{-value} > 0.05$) even if the efficiency indicates a slight underestimation of the target. For the mantle, the $2 \text{ ng} \cdot \mu\text{L}^{-1}$ is the maximum concentration that does not alter the linear 10-fold dilution curve ($E = 92.1 \%$, ANCOVA, $p\text{-value} > 0.05$). The curve corresponding to $5 \text{ ng} \cdot \mu\text{L}^{-1}$ shows non-significant difference with the standard but is discarded due to a lower concentration threshold for *P. olseni* ($2 \text{ ng} \cdot \mu\text{L}^{-1}$). The host tissue gDNA concentration of $2 \text{ ng} \cdot \mu\text{L}^{-1}$ was the safest trade-off showing no difference with the standard. For the remaining tissue, the $2 \text{ ng} \cdot \mu\text{L}^{-1}$ is the maximum concentration of gDNA that does not alter the linear 10-fold dilution curve ($E = 95.7 \%$; ANCOVA, $p\text{-value} > 0.05$).

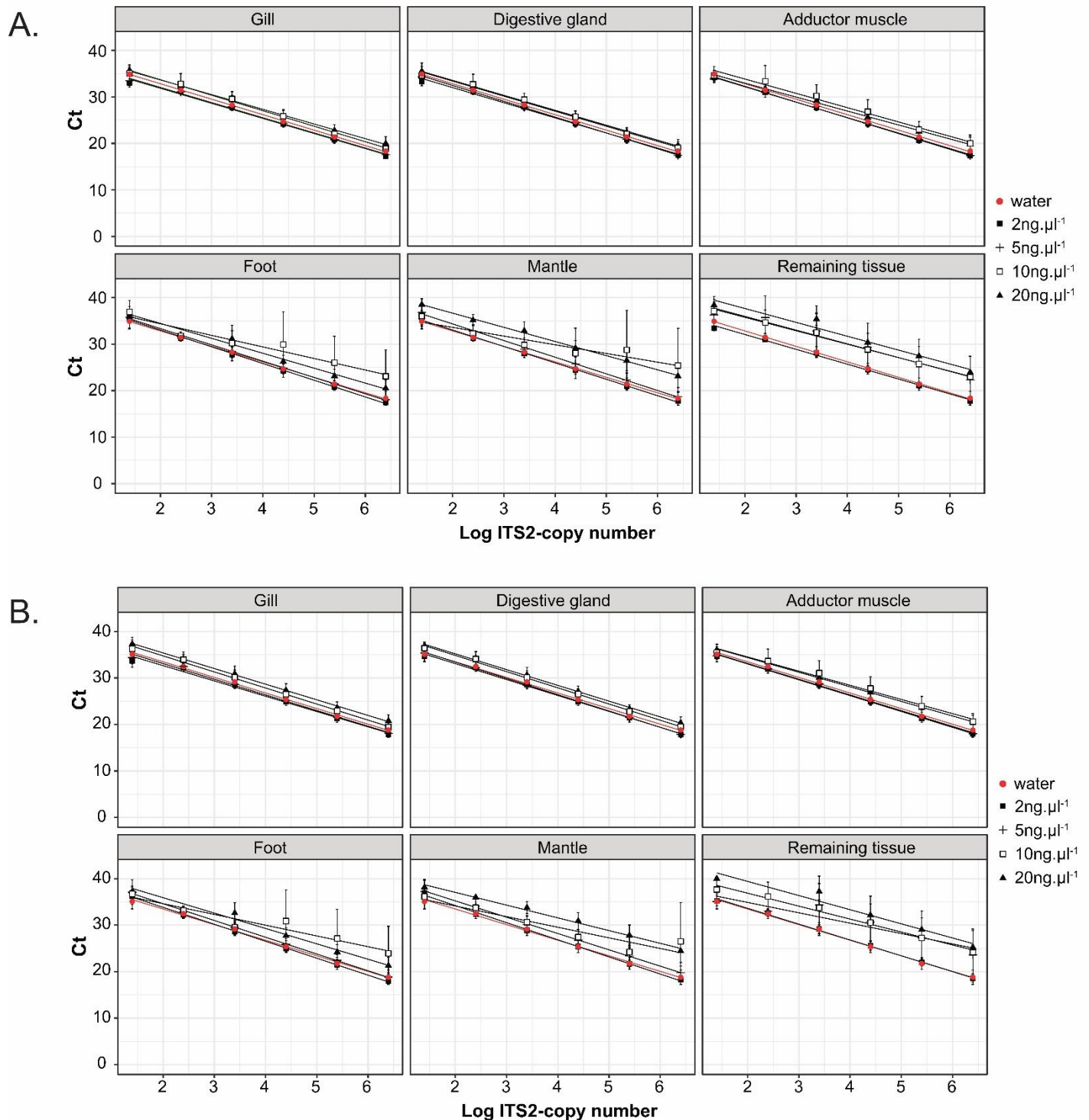


Figure 4. Sensitivity and inhibitory effects induced by four concentrations of gDNA from the six different types of tissue samples (gill, digestive gland, adductor muscle, foot, mantle and remaining tissue). The sensitivity of the real-time duplex PCR is represented for six (A) *P. olseni*-plasmids and (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.

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 are cumulated and compared with the RFTM infection intensities.

Based on the previous results described above, only Ct values within the standard range of 2.5×10^1 to 2.5×10^6 total copies are considered as positive. Therefore, all values below this range are considered as unquantifiable. No samples are detected above 2.5×10^6 total copies. The total prevalence of *Perkinsus* estimated by RFTM assay and qPCR assay is 74 % representing a total of 37 infected clams over the 50 clams tested (Table 3). Prevalence of *P. olseni* single-infection represents 68 % (34 clams) of sampled individuals whereas the co-infection represents solely 6 % (3 clams). Furthermore, among the co-infected hosts (n=3), the *Perkinsus* spp. mean infection intensity is $1.37 \times 10^4 \pm 2.14 \times 10^4$ cells. g⁻¹ of wet tissue. Based on paired and unpaired prevalences from both RFTM and qPCR assays, the concordance is estimated at 88 % and the discordance at 12 % (Table 4). The three discordant individuals, positive with RFTM assay and negative with qPCR assay, show very light infection intensities ranging from 8.1 cells.g⁻¹ of wet tissue to 6.0×10^2 cells.g⁻¹ of wet tissue.

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 (%)				
				<i>Perkinsus</i> sp.	<i>P. olseni</i>	<i>P. chesapeaki</i>	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		Concordance	a+d/n	88 %
		positive	negative			
Duplex qPCR assay	positive	34 (a)	3 (b)			
	negative	3 (c)	10 (d)			

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To determine the relationship between both quantitative methods, only positive clams in RFTM and qPCR were compared (Figure 5). A linear regression was determined for the qPCR-infection intensities and the RFTM-infection intensities relationship ($y=0.60x+4.36$, adjusted- $R^2=0.61$, $n=34$). A significant Pearson's coefficient is also obtained for the qPCR-infection intensities and the RFTM-infection intensities ($r=0.79$, $p\text{-value}<0.001$) showing a positive correlation. The infection intensity (in cells. g^{-1} of wet tissue) for each individual is evaluated from qPCR assay following the relationship described before ($y=0.60x+4.36$; Figure 5). A discrepancy from the linear regression line is observed for low infection values (below 100 cells. g^{-1} of wet gill). To optimise the parasite detection, negative individuals and mismatch individuals were discarded. Consequently, the intercept of this linear relationship is not equal to zero which introduce an overestimation of the infection intensity. The mean infection intensity estimated from qPCR assay is $3.35 \times 10^5 \pm 8.55 \times 10^5$ cell. g^{-1} of wet tissue. Separately, mean infection intensity for *P. olseni* is estimated at $1.37 \times 10^4 \pm 2.14 \times 10^4$ cells. g^{-1} of wet tissue and *P. chesapeaki* at $2.50 \times 10^1 \pm 2.69 \times 10^1$ cells. g^{-1} of wet tissue. The mean proportion of *P. olseni* represents $95 \% \pm 5\%$.

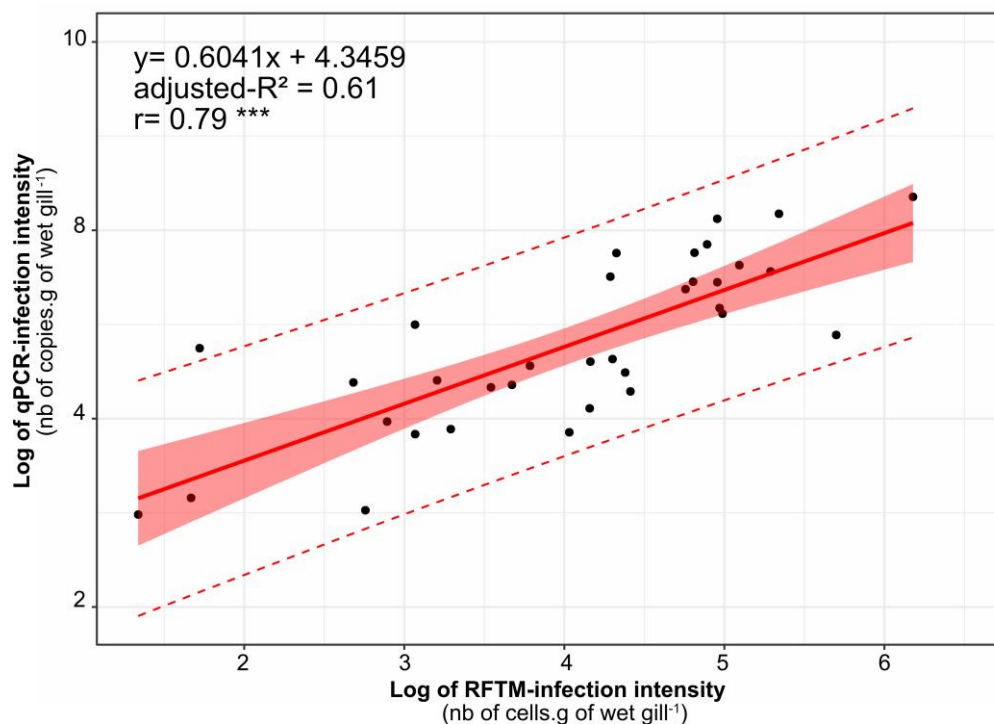


Figure 5. Linear regression between infection intensities determined by RFTM assay (nb. of cells.g of wet gill⁻¹) and by qPCR assay (nb. of copies.g of wet gill⁻¹). The infection intensities determined by RFTM are based on the global counting of *Perkinsus* sp. hypnospores. The infection intensities 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.

4. Discussion

4.1. The RFTM assay lacks of specificity

Almeida *et al.* (1999) suggested that planktonic organisms like dinoflagellates could lead to positive results in RFTM highlighting a lack of genus-specificity when used on environmental samples. This standard methodology also appears to be inappropriate for some members of the *Perkinsus* genus especially for the species *P. qugwadi* which does not form hypnospores in RFTM and may remain unnoticed for diagnosis (Itoh *et al.*, 2013). Finally, it does not distinguish between *Perkinsus* species because hypnospores show any distinctive morphological trait. Using only this methodology allows to estimate the intensity of a global *Perkinsus* infection that may lead to hide cryptic co-infection whereas it is a common configuration as it was described for *P. marinus* and *P. chesapeaki* in Chesapeake Bay (Reece *et al.*, 2008).

Using RFTM methodology, we observed 74% of prevalence with a strong infection intensity of $9.04 \times 10^4 \pm 2.24 \times 10^5$ cells.g⁻¹ of wet tissue which is coherent with the results obtained by Dang *et al.* (2010) in Arcachon Bay in November 2006 and 2007 where prevalences were 75% and 70% and infection intensities of 1.58×10^5 and 7.94×10^4 cells.g⁻¹ of wet tissue respectively. However, the diversity of *Perkinsus* species occurring in Arcachon Bay is yet not resolved. From a previous pre-sampling campaign carried out in October 2017 in the Arcachon bay (data not shown), *Perkinsus* culture cell lines were established from gill clam samples. The genetic diversity targeting the ITS1-5S-ITS2 rDNA region revealed that 17 cultures (n= 22) were affiliated to *P. olseni* with 100 % of identity (627 bp) while 5 cultures were affiliated to *P. chesapeaki* with 97 % to 100 % of identity (639 bp). All these results are congruent with previously studies showing the co-occurrence of these two *Perkinsus* species in European coastal environments (Arzul *et al.*, 2012; Ramilo *et al.*, 2016). Hence, the *in situ* occurrence of these cryptic infections in the Arcachon bay has required the development of non-culture based method as the duplex qPCR allowing the co-detection of these 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 proved to be successful for the diagnose of *in situ* infection by protists, virus 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). The detection of Perkinsosis was monitored by two types of real-time PCR chemistry, the SYBR Green (Audemard *et al.*, 2004; Ulrich *et al.*, 2007) and the 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 ran in this study, Ct values are close 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 close number of ITS2 copy in their genome 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 molecular multiplexing approach shows a rising interest in environments where multiple-species infection seems to be the rule (Bass *et al.*, 2019). The duplex qPCR method elaborated in this study demonstrates low qPCR inter-plates variations (CV% from 5 % to 8 %, Table 2) testifying of 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-standards (from 2.5×10^1 to 2.5×10^6 total copies) and for Perkinsosis, which may sometimes hide cryptic infections.

4.3. PCR inhibitors contained in biological tissue is a very common issue linked to qPCR assays

Inhibition effects are recognized issues for parasite detection within host samples (Audemard *et al.*, 2004, 2006). Organic and inorganic compounds present in the host matrix and extracted with gDNA may compromise the efficiency of DNA polymerases and ultimately produce a false negative or erroneous Ct value (De Faveri *et al.*, 2009). By producing standard curves supplemented with different concentrations of extract of organs gDNA, we show here that PCR

inhibitors are present in some clam compartments but can be resolved by adjusting matrix concentration.

In this study, quantification of plasmids standards (a useful and accurate proxy of *Perkinsus*) was tested with six different concentrations of gDNA organs (gills, digestive gland, foot, adductor muscle, mantle and the remaining tissue). For both *Perkinsus* species, gill and digestive gland gDNA extracts do not contain inhibitory compounds in the range of the qPCR detection as already described for gill tissue by Umeda and Yoshinaga (2012). Thus, for these two types of tissue, the gDNA concentration may easily reach $20 \text{ ng}\cdot\mu\text{L}^{-1}$ within the qPCR framework described above to optimise the *Perkinsus* detection and avoid qPCR saturation. Conversely, the adductor muscle and the foot on the one hand and the mantle and the remaining tissue on the other show an optimal amplification for host gDNA concentration of $5 \text{ ng}\cdot\mu\text{L}^{-1}$ and $2 \text{ ng}\cdot\mu\text{L}^{-1}$ respectively. These results are surprising because contrary to clams, the mantle and the rectum tissue from oysters showed to be neutral on the qPCR reaction (De Faveri *et al.*, 2009). Low load of organ extract gDNA, e.g. $2 \text{ ng}\cdot\mu\text{L}^{-1}$, decreases the detection limit of the parasite compared to a higher load. Thus, for organ like mantle or remaining tissue, the prevalence of *Perkinsus* parasite may be underestimated in case of low infection intensity. The thresholds mentioned in this study are basic recommendations for duplex TaqMan-based real-time PCR applied on further studies of the parasite dynamics in the Manila clam. These values are specific to the Manila clam and should be adjusted for each biological model and targeted organ. In spite of inter-individual variability between the three non-infected reference Manila clams used for the inhibitory test, a very clear inhibition trend related to the type of clam organ is observed. This kind of observation testify of the global utilisation of the gill for routine diagnostic for molecular methods including PCR and qPCR, which are a non-inhibited and easy proxy of *Perkinsus* infection.

4.4. Correlation between RFTM methodology and molecular tool: 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 2.5×10^1 to 2.5×10^2 total copies. For the other standard-points (i.e. from 2.5×10^2 to 2.5×10^6 total copies), the higher infection intensities enable a more homogeneous repartition of parasites in the gill and therefore a better qPCR detection. Most of the positive individuals (32 of 34 paired-positive Manila clams) are localised in the range of 2.5×10^2 to 2.5×10^6 total copies leading to a good correlation between RFTM and qPCR assay quantification.

In this study, there is no difference of prevalence (74% with 88% of concordance). However, we observe that the mean infection intensity for the RFTM method ($9.04 \times 10^4 \pm 2.24 \times 10^5$ cells. g^{-1} of wet tissue) seems lower than the qPCR assay ($3.35 \times 10^5 \pm 8.55 \times 10^5$ 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: 1) the discrepancy in the ploidy treatment of the two methods, the qPCR assay determines molecularly a total number of copies in trophozoites and schizonts while the RFTM assay determines microscopically a total number of hypnospores; and 2) the uncertainty of a full transformation of trophozoites and schizonts into hypnospores, not all trophozoites (dividing, immature, mature or dead) may transform into an equal number of hypnospores when incubated in the thioglycollate medium. In this case, the RFTM method tends to underestimate the overall infection intensity. This underestimation can be accentuated for the lowest infection intensities (e.g. for 8.1 to 6.0×10^2 cells. g^{-1} of wet tissue in this study). The physiological status of the parasite and the absence of hypnospore formation can have a much greater impact on the estimation of low infection intensities than intensities comprising thousands of cells per gram of tissue. In addition, the infection intensities of the duplex qPCR are indirectly estimated by a mathematical relationship ($y=0.6041x+4.3459$; adjusted- $R^2 = 0.61$) relying on the RFTM counting method. Moreover, this linear equation has a positive value of intercept which impacts strongly lowest values of infection.

Finally, the molecular method measures a total amount of DNA copies regardless of the physiological stage of *Perkinsus* cells while the traditional method allows the counting of trophozoites that are able to transform into hypnospores. Given their respective biases, it is not surprising that qPCR infection intensity values appear to be higher than those of the RFTM assay. Even if the RFTM method has bias that are often overlook in the literature, the duplex qPCR method is an interesting proxy which could complete the Perkinsosis diagnostic commonly used.

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

Relationship between both detection methods enabled us to evaluate the prevalence and intensity of infection of both parasites *P. olseni* and *P. chesapeaki* in a same host gill tissue sample. At the Lanton station, co-infection appears at very low frequency (6 %, n=3/50 individuals sampled). *P. olseni* largely dominates the cases of single-infection and remains preponderant in co-infection. Conversely, *P. chesapeaki* is much more discrete and with much lower intensities. We demonstrated that *P. olseni* invades more efficiently the gill with 95 % of occupation compared to *P. chesapeaki*. Indeed, *P. chesapeaki* represents only 5 % of the parasitic load in the co-infected clams. First evidence leads to believe that the distribution between *P. olseni* and *P. chesapeaki* would not be homogeneous within clam organs. Arzul *et al.* (2012) described in *R. 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 repartition could be very different depending on their interaction and their spatial and temporal dynamic (Cui *et al.*, 2018). The development of a duplex qPCR method allowed an easier detection and quantification of the co-infection phenomenon and the share of responsibility of each parasite in the infected host. It is therefore now critical to consider the whole-body compartment in a host to reach the most accurate diagnostic of Perkinsosis.

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 the quantification can be overestimated. The RFTM seems to underestimate results on the infection intensity of *Perkinsus* sp. but is not enough informative at the species level. The standard culture method and the molecular method are not conflicting approach and depend on level of accuracy required to address the *Perkinsus* infection issue. 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

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management of potential valuable hosts living in sympatry. The coupling of both methods is a powerful approach to fully understand the Perkinsosis dynamic and prevent the spread of these parasites in new hosts and environments.

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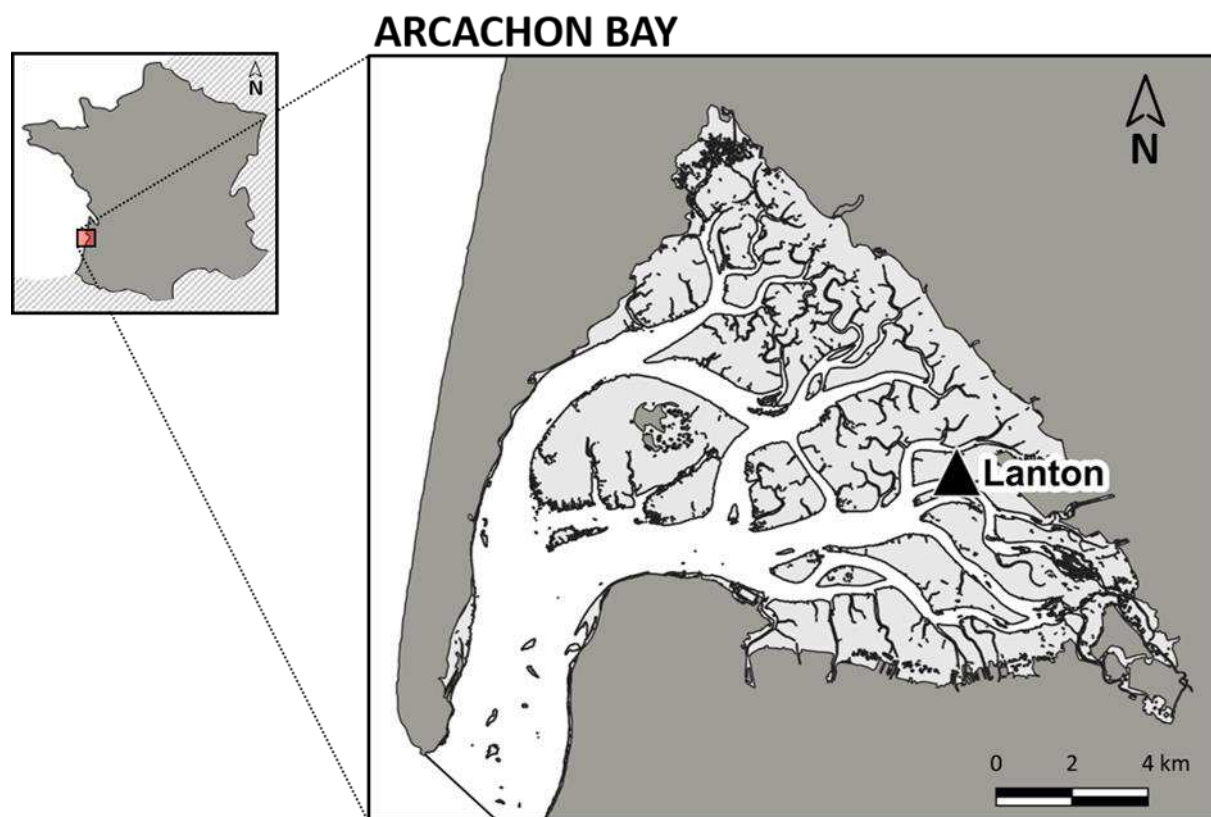


Figure S1. Map of the sampling site in Arcachon Bay (SW France, Atlantic coast, 44°41'60" N;1°10' W) indicating the collecting point at Lanton.

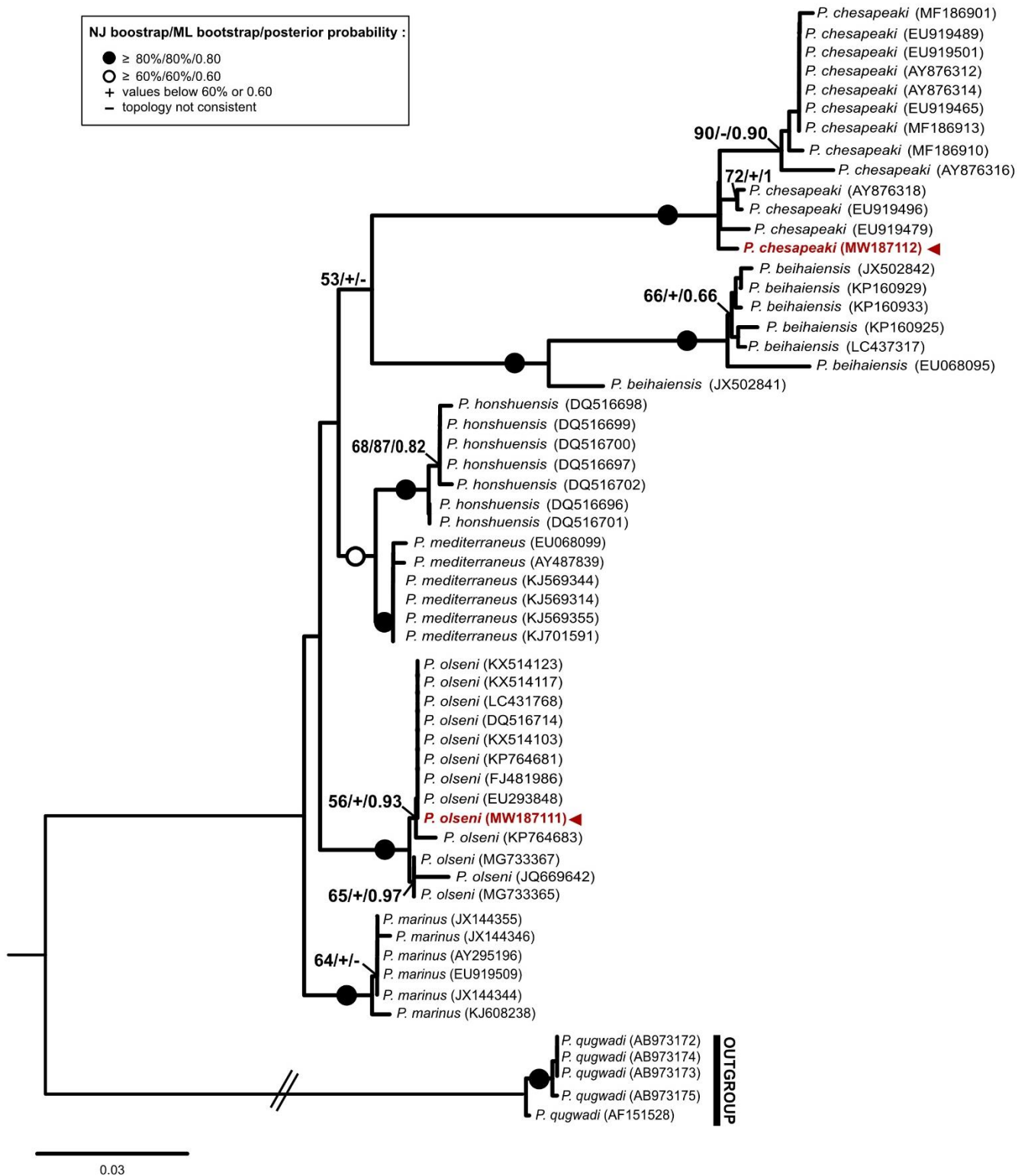


Figure S2. Neighbour joining (NJ) tree of *Perkinsus* genus diversity using the ITS1-5.8S-ITS2 region of the ribosomal cluster. The phylogeny was calculated from 57 taxa and 633 characters alignment position. NJ distances (1000 replicates), Maximum Likelihood (ML) bootstraps (1000 replicates) and Bayesian posterior probability (2 000 000 generations, HKY+ Γ 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.

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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 diagnostic	50	74	9.07x10 ⁴ ± 2.24x10 ⁵
			Culture	5	100	nd

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Table S2. GenBank ITS1-5.8S-ITS2 sequence region belonging to *Perkinsus* genus used in phylogenetic analysis to affiliate *P. olseni* and *P. chesapeakei* sequences isolated from the Arcachon bay

Accession no.	Class	Order	Genus	Species	Strain/Clone	Reference
JX144346	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>marinus</i>	60_2	(da Silva <i>et al.</i> , 2013)
KJ608238	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>marinus</i>	70_3_C_c5	(da Silva <i>et al.</i> , 2014)
JX144355	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>marinus</i>	69	(da Silva <i>et al.</i> , 2013)
EU919509	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>marinus</i>	PXBICv25-B9-C5	(Reece <i>et al.</i> , 2008)
AY295196	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>marinus</i>	SC2_4_7	(Brown <i>et al.</i> , 2004)
JX144344	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>marinus</i>	60	(da Silva <i>et al.</i> , 2013)
KJ569344	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>mediterraneus</i>	H08_3_06	(Valencia <i>et al.</i> , 2014)
AY487839	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>mediterraneus</i>	D10	(Casas <i>et al.</i> , 2004)
KJ569314	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>mediterraneus</i>	H03_3_04	(Valencia <i>et al.</i> , 2014)
KJ569355	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>mediterraneus</i>	H03_28_A3	(Valencia <i>et al.</i> , 2014)
EU068099	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>mediterraneus</i>	Jer2_50	(Casas <i>et al.</i> , 2008)
KJ701591	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>mediterraneus</i>	12	(Ramilo <i>et al.</i> , 2015)
DQ516701	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>honshuensis</i>	Mie3gH8_3	(Dungan and Reece, 2006)
DQ516696	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>honshuensis</i>	Mie3g_2	(Dungan and Reece, 2006)
DQ516702	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>honshuensis</i>	Mie3gH8_4	(Dungan and Reece, 2006)
DQ516699	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>honshuensis</i>	Mie3gH8_1	(Dungan and Reece, 2006)
DQ516700	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>honshuensis</i>	Mie3gH8_2	(Dungan and Reece, 2006)
DQ516698	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>honshuensis</i>	Mie3g_4	(Dungan and Reece, 2006)
DQ516697	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>honshuensis</i>	Mie3g_3	(Dungan and Reece, 2006)
JX502841	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>beihaiensis</i>	138	(Sabry <i>et al.</i> , 2013)
KP160933	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>beihaiensis</i>	CBMA287	(Queiroga <i>et al.</i> , 2015)
LC437317	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>beihaiensis</i>	Koitogawa_4	Itoh <i>et al.</i> 2019 unpublished
KP160925	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>beihaiensis</i>	CBMA92	(Queiroga <i>et al.</i> , 2015)
KP160929	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>beihaiensis</i>	CBMA142	(Queiroga <i>et al.</i> , 2015)
JX502842	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>beihaiensis</i>	34	(Sabry <i>et al.</i> , 2013)
EU068095	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>beihaiensis</i>	QZ0649	(Moss <i>et al.</i> , 2008)
AB973173	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>qugwadi</i>	8302-1	(Itoh <i>et al.</i> , 2013)
AB973174	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>qugwadi</i>	8302-11	(Itoh <i>et al.</i> , 2013)
AB973175	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>qugwadi</i>	8302-51	(Itoh <i>et al.</i> , 2013)
AB973172	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>qugwadi</i>	6675-2	(Itoh <i>et al.</i> , 2013)
AF151528	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>qugwadi</i>	-	Hervio <i>et al.</i> 1999 unpublished

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EU919479	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>chesapeaki</i>	EBNPMb2-B5-D12	(Reece <i>et al.</i> , 2008)
EU919489	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>chesapeaki</i>	PXBIMa10-D10-E4	(Reece <i>et al.</i> , 2008)
EU919501	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>chesapeaki</i>	YRKCMb1-G2-G8	(Reece <i>et al.</i> , 2008)
AY876312	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>chesapeaki</i>	ATCC 50807	(Burreson <i>et al.</i> , 2005)
AY876314	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>chesapeaki</i>	ATCC 50807	(Burreson <i>et al.</i> , 2005)
EU919465	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>chesapeaki</i>	CRBSTp9	(Reece <i>et al.</i> , 2008)
EU919496	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>chesapeaki</i>	PXSATp6-A7-A8	(Reece <i>et al.</i> , 2008)
AY876318	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>chesapeaki</i>	ATCC PRA-65	(Burreson <i>et al.</i> , 2005)
AY876316	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>chesapeaki</i>	ATCC PRA-65	(Burreson <i>et al.</i> , 2005)
MF186901	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>chesapeaki</i>	A9-1	(Reece <i>et al.</i> , 2017)
MF186913	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>chesapeaki</i>	A5_4	(Reece <i>et al.</i> , 2017)
MF186910	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>chesapeaki</i>	A7_2	(Reece <i>et al.</i> , 2017)
JQ669642	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>olseni</i>	05067_3P2/1_6	(Arzul <i>et al.</i> , 2012)
KX514117	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>olseni</i>	MS2-2	Cho <i>et al.</i> 2018 unpublished
KX514123	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>olseni</i>	SS2-2	Cho <i>et al.</i> 2018 unpublished
LC431768	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>olseni</i>	shoG	Imajoh <i>et al.</i> 2018 unpublished
DQ516714	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>olseni</i>	Mie13v_8	(Dungan and Reece, 2006)
MG733367	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>olseni</i>	PM51DH1	(Shamal <i>et al.</i> , 2018)
MG733365	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>olseni</i>	PM45EL4	(Shamal <i>et al.</i> , 2018)
KP764683	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>olseni</i>	8	(Ramilo <i>et al.</i> , 2016) Cho <i>et al.</i> 2018
KX514103	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>olseni</i>	WD4-2	unpublished
KP764681	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>olseni</i>	6	(Ramilo <i>et al.</i> , 2016) Elandaloussi <i>et al.</i> , 2008
FJ481986	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>olseni</i>	Rp1	unpublished (Elandaloussi <i>et al.</i> , 2009)
EU293848	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>olseni</i>	-	(Elandaloussi <i>et al.</i> , 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	<i>Perkinsus chesapeaki</i>		no match sequences		
	31276	<i>Perkinsus marinus</i>	F:5, R:5	<i>Perkinsus marinus</i> ATCC 50983 hypothetical protein, mRNA	XM_002787768.1	1 082
	259652	<i>Perkinsus mediterraneus</i>		no match sequences		
	1074429	<i>Perkinsus beihaiensis</i>	F:1, R:4	<i>Perkinsus beihaiensis</i> isolate 138 et 31	JX502841.1/JX502840.1	83
	386307	<i>Perkinsus honshuensis</i>		no match sequences		
	103982	<i>Parvilucifera</i> spp.		no match sequences		
			F:4, R:4	<i>Plasmodium knowlesi</i> strain H SICA antigen (fragment) partial mRNA	XM_002259474.1	2 294
	5794	Apicomplexa	F:5, R:4	<i>Plasmodium cynomolgi</i> genome assembly, chromosome: 7	LT841385.1	1 027
			F:5, R:4	<i>Gregarina niphandrodes</i> peptidase partial mRNA	XM_011133033.1	2 460
		2864	Dinoflagellates		no match sequences	
	31291	Haplosporidians		no match sequences		
	33674	Thraustochytridae		no match sequences		
PchesITS2_F/PchesITS2_R	32597	<i>Perkinsus olseni</i>		no match sequences		
	31276	<i>Perkinsus marinus</i>		no match sequences		
	259652	<i>Perkinsus mediterraneus</i>		no match sequences		
	1074429	<i>Perkinsus beihaiensis</i>		no match sequences		
	386307	<i>Perkinsus honshuensis</i>		no match sequences		
	103982	<i>Parvilucifera</i> spp.		no match sequences		
	5794	Apicomplexa	F:5, R:5	<i>Neospora caninum</i> Liverpool, chromosome chrXI, complete genome	LN714486.1	3 944
	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: <i>Quercus suber</i> beta-mannosidase A-like (LOC112014742), mRNA	36.2	36.2	90%	8.3	100%	XM_024047133.1
<i>Rhodococcus</i> sp. DMU1 chromosome, complete genome	34.2	34.2	85%	33	100%	CP050952.1
<i>Monaibacterium</i> sp. ALG8 chromosome, complete genome	34.2	34.2	85%	33	100%	CP049811.1
<i>Halogeometricum borinquense</i> strain wsp4 chromosome, complete genome	34.2	34.2	85%	33	100%	CP048739.1
<i>Salmo trutta</i> genome assembly, chromosome: 12	34.2	34.2	85%	33	100%	LR584441.1
<i>Salmo trutta</i> genome assembly, chromosome: 29	34.2	34.2	85%	33	100%	LR584418.1
<i>Bos mutus</i> isolate yakQH1 chromosome 22	34.2	34.2	85%	33	100%	CP027090.1
<i>Ovis canadensis canadensis</i> isolate 43U chromosome 19 sequence	34.2	34.2	85%	33	100%	CP011904.1
<i>Rhodococcus aetherivorans</i> strain IcdP1, complete genome	34.2	34.2	85%	33	100%	CP011341.1
PREDICTED: <i>Callorhinchus milii</i> cytosolic phospholipase A2 gamma-like (LOC103190768), transcript variant X1, mRNA	34.2	34.2	85%	33	100%	XM_007911657.1
* <i>Perkinsus</i> sp. : KM983404, LC524156, AF522321, U07699, U07698, KM983403, KM983402, KM983401	99.52% to 100% : <i>P. olseni</i>					

B.

10 Best hits for *P. chesapeaki* probe sequence

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
<i>Arabis alpina</i> genome assembly, chromosome: 5	40.1	40.1	90%	0.53	100%	LT669792.1
<i>Arabis alpina</i> genome assembly, chromosome: 4	40.1	152	90%	0.53	100%	LT669791.1
<i>Arabis alpina</i> genome assembly, chromosome: 2	40.1	199	90%	0.53	100%	LT669789.1
<i>Microbacterium</i> sp. SGAir0570 chromosome, complete genome	36.2	36.2	81%	8.3	100%	CP027929.1
<i>Cellulomonas shaoxiangyii</i> strain Z28 chromosome, complete genome	36.2	36.2	81%	8.3	100%	CP039291.1
<i>Halomonas alkaliphila</i> X3, complete sequence	36.2	36.2	81%	8.3	100%	CP024811.1
<i>Paenibacillus lautus</i> strain E7593-69 chromosome, complete genome	36.2	36.2	81%	8.3	100%	CP032412.1
<i>Rubrobacter indicocéani</i> strain SCSIO 08198 chromosome	36.2	36.2	81%	8.3	100%	CP031115.1
<i>Kocuria rhizophila</i> strain NCTC8340 genome assembly, chromosome: 1	36.2	36.2	81%	8.3	100%	LR134409.1
<i>Kocuria</i> 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, 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 98.93% to 100% : *P. chesapeaki*

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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⁻¹) tested on both *P. olsenii*-plasmidic and *P. chesapeakei*-plasmidic serial dilutions. Red highlights represent best parameters for *P. olsenii*; grey highlights represent best parameters for *P. chesapeakei*.

Species	Type of organ	A concentration	Equation curve	R ²	ANCOVA (F)	p-value	Efficiency (10 ^(-1/slope) × 100%)
<i>P. olsenii</i>	Gill	2	-3,34x + 38,77	0,99	0,00	ns	99,9%
<i>P. olsenii</i>	Gill	5	-3,13x + 38,82	0,99	0,01	ns	108,6%
<i>P. olsenii</i>	Gill	10	-3,37x + 40,57	0,93	0,10	ns	97,9%
<i>P. olsenii</i>	Gill	20	-3,32x + 39,97	0,96	1,85	ns	100,3%
<i>P. olsenii</i>	Digestive gland	2	-3,32x + 38,83	0,99	0,01	ns	100,3%
<i>P. olsenii</i>	Digestive gland	5	-3,43x + 39,38	1,00	0,52	ns	95,9%
<i>P. olsenii</i>	Digestive gland	10	-3,28x + 40,11	0,93	0,09	ns	101,7%
<i>P. olsenii</i>	Digestive gland	20	-3,31x + 39,75	0,98	0,82	ns	100,4%
<i>P. olsenii</i>	Adductor muscle	2	-3,40x + 39,09	1,00	0,22	ns	97,1%
<i>P. olsenii</i>	Adductor muscle	5	-3,36x + 39,00	0,99	0,04	ns	98,6%
<i>P. olsenii</i>	Adductor muscle	10	-3,13x + 40,27	0,84	2,88	ns	108,7%
<i>P. olsenii</i>	Adductor muscle	20	-3,00x + 38,97	0,97	5,54	*	115,4%
<i>P. olsenii</i>	Foot	2	-3,57x + 40,22	0,99	4,47	*	90,6%
<i>P. olsenii</i>	Foot	5	-3,51x + 40,36	0,97	1,61	ns	92,8%
<i>P. olsenii</i>	Foot	10	-2,54x + 39,70	0,47	8,43	**	147,3%
<i>P. olsenii</i>	Foot	20	-3,26x + 41,27	0,91	17,59	***	102,7%
<i>P. olsenii</i>	Mantle	2	-3,53x + 40,07	0,99	2,43	ns	92,0%
<i>P. olsenii</i>	Mantle	5	-3,57x + 41,46	0,94	2,28	ns	90,6%
<i>P. olsenii</i>	Mantle	10	-1,83x + 37,25	0,24	16,04	***	252,2%
<i>P. olsenii</i>	Mantle	20	-3,05x + 42,76	0,90	2,84	ns	112,9%
<i>P. olsenii</i>	Remaining tissue	2	-3,19x + 38,46	0,99	1,06	ns	105,6%
<i>P. olsenii</i>	Remaining tissue	5	-2,99x + 42,07	0,55	6,11	*	115,9%
<i>P. olsenii</i>	Remaining tissue	10	-2,89x + 41,55	0,68	2,16	ns	121,7%
<i>P. olsenii</i>	Remaining tissue	20	-3,07x + 44,28	0,93	8,71	**	111,9%
<i>P. chesapeakei</i>	Gill	2	-3,38x + 39,71	0,99	0,00	ns	97,4%
<i>P. chesapeakei</i>	Gill	5	-3,44x + 40,18	0,98	0,15	ns	95,4%
<i>P. chesapeakei</i>	Gill	10	-3,50x + 41,89	0,94	0,65	ns	93,1%
<i>P. chesapeakei</i>	Gill	20	-3,36x + 42,12	0,95	0,01	ns	98,3%
<i>P. chesapeakei</i>	Digestive gland	2	-3,47x + 40,18	0,99	0,42	ns	94,0%
<i>P. chesapeakei</i>	Digestive gland	5	-3,53x + 40,52	1,00	1,01	ns	92,2%
<i>P. chesapeakei</i>	Digestive gland	10	-3,51x + 41,97	0,95	0,92	ns	92,5%
<i>P. chesapeakei</i>	Digestive gland	20	-3,39x + 41,97	0,96	0,01	ns	97,2%
<i>P. chesapeakei</i>	Adductor muscle	2	-3,43x + 39,94	0,99	0,12	ns	95,6%
<i>P. chesapeakei</i>	Adductor muscle	5	-3,41x + 40,03	0,99	0,06	ns	96,3%
<i>P. chesapeakei</i>	Adductor muscle	10	-3,09x + 40,84	0,85	5,47	*	110,5%
<i>P. chesapeakei</i>	Adductor muscle	20	-3,15 + 40,69	0,97	2,34	ns	107,8%
<i>P. chesapeakei</i>	Foot	2	-3,64x + 41,08	0,99	2,22	ns	88,3%
<i>P. chesapeakei</i>	Foot	5	-3,61x + 41,83	0,95	1,98	ns	89,3%
<i>P. chesapeakei</i>	Foot	10	-2,32x + 39,39	0,40	24,56	***	169,6%
<i>P. chesapeakei</i>	Foot	20	-3,37x + 42,98	0,98	16,08	***	98,2%
<i>P. chesapeakei</i>	Mantle	2	-3,53x + 40,80	0,99	4,60	ns	92,1%
<i>P. chesapeakei</i>	Mantle	5	-3,51x + 42,26	0,93	1,50	ns	92,6%
<i>P. chesapeakei</i>	Mantle	10	-2,15x + 38,31	0,45	11,29	**	192,3%
<i>P. chesapeakei</i>	Mantle	20	-2,75x + 42,59	0,89	12,46	***	130,8%
<i>P. chesapeakei</i>	Remaining tissue	2	-3,43x + 40,56	0,98	0,12	ns	95,7%
<i>P. chesapeakei</i>	Remaining tissue	5	-2,45x + 40,49	0,38	39,09	***	156,4%
<i>P. chesapeakei</i>	Remaining tissue	10	-2,80x + 42,53	0,64	5,65	*	127,4%
<i>P. chesapeakei</i>	Remaining tissue	20	-3,26x + 46,87	0,91	13,55	***	102,5%

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Chapitre 2.2 : Occurrence et distribution de deux parasites invasifs émergents, *Perkinsus olsenii* et *P. chesapeaki*, de l'échelle individuelle à l'échelle locale (bassin d'Arcachon, France)

Avant-propos

Le **Chapitre 2.1** nous a permis de poser les bases méthodologiques pour l'étude *in situ* de la co-infection entre *P. olsenii* et *P. chesapeaki*. Nous avons constaté que la réaction de PCR pouvait être inhibée en fonction de la nature de l'organe hôte. Le manteau et le reste du corps mou ont un effet inhibiteur élevé à partir du seuil de concentration de l'ADN génomique (ADNg) de l'hôte de $2 \text{ ng} \cdot \mu\text{L}^{-1}$, le muscle adducteur et le pied ont une inhibition intermédiaire pour une concentration supérieure à $5 \text{ ng} \cdot \mu\text{L}^{-1}$ et enfin, les branchies et la glande digestive ne montrent aucune inhibition pour une concentration maximum de $20 \text{ ng} \cdot \mu\text{L}^{-1}$. Ainsi, lors des premières détections réalisées sur la branchie, qui est un organe de diagnostic recommandé par l'O.I.E, des infections cryptiques entre ces deux parasites ont été révélées contrairement au diagnostic RFTM. De façon générale, cette méthode s'est avérée être reproductible, très efficace sur les intensités d'infection modérées à fortes et complémentaire à la méthode au RFTM. Au cours de ce **Chapitre 2.2** nous proposons d'étudier la prévalence et la distribution des (co-)infections à *P. olsenii* et *P. chesapeaki* au sein des palourdes du bassin d'Arcachon en adoptant approche compartimentée du corps de l'hôte. Lors d'un second échantillonnage réalisé en 2018, nous avons échantillonné les populations de palourdes japonaises au sein de cinq stations contrastées dans le bassin d'Arcachon. Afin d'étudier la répartition des différents types d'infection, les palourdes ont été disséquées suivant six organes : les branchies, la glande digestive, les muscles adducteurs, le pied, le manteau et le reste du corps mou. Ce **Chapitre 2.2** a pour but d'obtenir une vision générale du fonctionnement de ces infections cryptiques à l'échelle de la population hôte et du bassin d'Arcachon. Nous allons ainsi caractériser les (co-)infections à *P. olsenii* et *P. chesapeaki* en intégrant les paramètres physiologiques de l'hôte et les paramètres environnementaux qui peuvent moduler ces interactions.

Occurrence and distribution of two emerging pathogens, *Perkinsus olseni* and *Perkinsus chesapeaki* (Perkinsozoa, Alveolata), from individual host to local scale (Arcachon bay, France).

Abstract

Perkinsus species are well-known parasitic micro-eucaryotes infecting wide range of mollusc species worldwide. Co-infections between two *Perkinsus* species, *P. olseni* and *P. chesapeaki*, were sporadically described in Europe in clam populations. Here, we study the *P. olseni* single-infection, *P. chesapeaki* single-infection and co-infection between both parasitic species distributed across five contrasted stations in the Arcachon bay (France). Manila clams sampled were dissected into six organs to investigate the distribution of different types of infection. Each sample was analysed using a qPCR duplex methodology allowing simultaneous *in situ* detection of both parasitic species. We highlighted relevant global *Perkinsus* spp. prevalences of co-infection ranging from 12 to 36% across four stations of the Arcachon bay. Interestingly, *P. olseni* mono-infection dominated largely prevalences (46 to 86 %) with high intensities of infection (7.2 to 8.5 log-nb of copies. g⁻¹ of wet tissue of Manila clams) suggesting that infection is driven by abiotic characteristics of stations and physiological factors of host. Conversely, *P. chesapeaki* mono-infections were low (2% and 12 %) and localised mostly in two stations. Finally, we revealed that co-infections were punctual and occurred in one or two organs within each co-infected individual. Within these co-infected organs in every sampling station, *P. olseni* largely dominated the global parasite load. Hence, co-infections might be impacted by biotic and abiotic factors suggesting different portal of entry for *P. chesapeaki* (co-detection in gill or adductor muscle or digestive gland, mantle and remaining tissue). Surprisingly, *P. chesapeaki* was almost exclusively found in co-infection suggesting that *P. olseni* may have a facilitating role as primary infection. This interaction may help *P. chesapeaki* to use *R. philippinarum* as a reservoir which does not represent preferred host. This ecological study demonstrated that the detection and quantification of *P. olseni* and *P. chesapeaki* is essential and timely to resolve cryptic infections and their consequences on hosts population across different environments.

1. Introduction

In a context of emergence of aquatic animal diseases, one host-one pathogen interactions have been considered, for a long time, as the golden rule with the paradigm "one parasite-one disease". However, it is now commonly agreed that co-infection by multiple parasites is more prevalent than previously thought (Petney and Andrews, 1998; Cox and Chappell, 2001). These associations are critical to understand disease dynamics and outcomes (Vayssier-Taussat *et al.*, 2014, 2015; Bass *et al.*, 2019). In medical and veterinary fields, co-occurrence of multiple pathogens have gained significant interest with reported studies on human (e.g. HIV-*Plasmodium falciparum*: Abu-Raddad *et al.*, 2006; *Plasmodium falciparum*-helminths: Brooker *et al.*, 2007; HIV-*Mycobacterium tuberculosis*: Teweldemedhin *et al.*, 2018) and animal health (e.g. *Theileria parva*-*Trypanosoma* spp.-strongyles in cattle: Thumbi *et al.*, 2014; multiple *Borrelia* species in ticks: Raileanu *et al.*, 2017). All these studies highlighted that results of co-infection are complex because it could lead independently to heterogeneous disease outcomes with the amplification or the suppression of the weight of one or more parasites (Johnson and Hoverman, 2012). These consequences may be mediated by direct competition for resources or space (ecological interactions) or indirect removal via the host immune system (immunological interactions) (Cox and Chappell, 2001; Graham, 2008). However, multiple infections have received little attention, so far, especially in marine ecosystems. One explanation is the difficulty to characterise micro-organisms, which have been often limited to culture and microscopy techniques. Recently the advent of molecular biology and environmental sequencing has revolutionised the concept of microbial diversity with the (re)-discovery and (re)-characterisation of many species of eukaryotic parasites (Moon-van der Staay *et al.*, 2001; Moreira and Lopez-Garcia, 2003; Chambouvet *et al.*, 2008). This has brought new insights in species definition and unravelled many cases of cryptic infectious agents (Putaporntip *et al.*, 2009; Chambouvet *et al.*, 2015). It is now urgent to tackle the fundamental question about the multiple infections to understand aquatic disease dynamics because, in marine ecosystems, mass mortality events (MME) keep increasing in prevalence and severity worldwide due to global changes (Harvell, 1999; Burge *et al.*, 2014).

Perkinsosis is an emerging disease of bivalve molluscs (e.g. clams and oyster) and gastropods (abalone) caused by the parasitic protists belonging to *Perkinus* genus (Alveolata). Heavily infected hosts show the presence of milky white nodules in gills mantle and foot, a disruption

of connectives tissue and epithelial cells, pale appearance of the digestive gland, reduction of the condition index and severe emaciation which all could drive to the host death (Ruano *et al.*, 2015). In Europe, *P. olseni* is regularly detected along the Atlantic and Mediterranean coasts (Ruano *et al.*, 2015) and have been associated with high mortality rate resulting in severe economic loss mainly in Portugal, Spain and along the Mediterranean coast (Da Ros and Canzonier, 1985; Ruano and Cachola, 1986; Azevedo, 1989; Pretto *et al.*, 2014). It has been hypothesised that *P. olseni* was accidentally co-introduced into European waters with its host the Manila clam (*Ruditapes philippinarum*) from Asia in the 1970s for aquaculture purpose (Le Borgne, 1996; Hine and Diggles, 2002). This hypothesis was supported by the low genetic diversity observed in *P. olseni* in Europe compare to those collected in Japan and in New Zealand (Vilas *et al.*, 2011). Hence *P. olseni* infection has a strong economic impact in Europe because this parasite infects one of the ten most valuable bivalves, the exotic Manila clams. The European production is estimated at 632 000 tons of Manila clams harvested in 2014 (FAO, 2018). However the situation is not so clear-cut because recent findings revealed that *P. olseni* is co-infecting in sympatry with congeneric species, *P. chesapeakei* in *Ruditapes decussatus* from Leucate lagoon, France (Arzul *et al.*, 2012) and Manila clams, *R. philippinarum*, from in the Arcachon bay (France) (Itoiz *et al.*, submitted). First detection of *P. chesapeakei* was in *Mya arenaria* from the Chesapeake Bay (McLaughlin and Faisal, 2000b). Since then, it has been detected in many other bivalves, e.g *Mercenaria mercenaria* (Reece *et al.*, 2008), *Tagelus plebeius* (Bushek *et al.*, 2008) or *Crassostrea rhizophorae* (Dantas Neto *et al.*, 2016), in South America, North America, Asia, and Europe (reviewed in Itoiz *et al.*, in prep). This parasitic species has also been found in association with *Perkinsus marinus* in oysters and clams from the Chesapeake Bay (Reece *et al.*, 2008). However, *P. chesapeakei* prevalences and infection intensities demonstrated a host preference for clams (Reece *et al.*, 2008; Itoiz *et al.* in prep). Regarding the history of *P. chesapeakei* in Europe, its introduction possibly occurred via its vectors the soft-shell clam, *Mya arenaria*, or the hard clam, *Mercenaria mercenaria* from the U.S.A (Arzul *et al.*, 2012). To date, no mortality event affiliated to *P. chesapeakei* has been recorded. Sporadic detections of *P. chesapeakei* in clams, mainly associated with *P. olseni*, does not show an important dynamic of invasion in these host populations. However, the detection of these two parasitic species occupying the same niche is enigmatic according to the competitive exclusion principle (Gause, 1934).

It is now timely to investigate ecological and biological mechanisms that could explain co-occurrence of two parasitic species belonging to *Perkinsus* genus in a context of emerging diseases. The aim of this study was to evaluate in Arcachon bay: (1) the diversity of *Perkinsus* species in Manila clam populations; (2) the distribution of single-infection and co-infection between *P. olseni* and *P. chesapeaki* across five contrasted sampling areas; and (3) the partitioning of host resource using a Taq-Man qPCR duplex methodology that allows detection and quantification of both parasitic species across organs of co-infected clams.

2. Material & methods

2.1. Sampling and clam conditioning

The Arcachon Bay (South West of France, Atlantic coast, 44°41'60" N; 1°10'W) is a mesotidal semi-sheltered lagoon of 180 km². This marine system is under oceanic (Atlantic tidal regime) and freshwater influence (Leyre River flow) encompassed by a 280 km² drainage basin. Representing up to 90% of total biomasses from muddy sediments (Bertignac *et al.*, 2001), the exotic Manila clam, *Ruditapes philippinarum*, represents today 300 to 500 tons of harvested resource each year despite a significant lack of recruitment and growth (de Montaudouin *et al.*, 2016). To date, the Perkinsosis disease is regularly detected in Manila clam populations but no mortality events have been reported (Dang *et al.*, 2010a; b; de Montaudouin *et al.*, 2016). Localisation in the bay and sediment type were determined by de Montaudouin *et al.* in 2010 for Andernos exclusively and Binias *et al.* in 2014 at higher scale. Here, we characterised stations as follows: Andernos as an intern station with fine sand; Lanton and Gujan as intern stations with muddy sand; IAO and Piquey as external oceanic stations with medium sand. Manila clams were sampled in the Arcachon bay on the 1st to 2nd of October 2017 and 7th to 8th of November 2018 in five contrasted intertidal sites: Lanton, Gujan, Andernos, Ile aux Oiseaux (IAO) and Piquey (Table 1).

Table 1. Supplementary informations on sampling sites in October 2017 and November 2018. n: total number of Manila clams sampled. Differences between mean infection intensities are tested by a non-parametric test of Kruskal-Wallis (df=4;H=14,6; p-value < 0.01) following by a non-parametric test of Dunn. Significant differences are indicated as follow : p-value < 0.05 ‘*’ and < 0.01 ‘***’.

Site	Sampling	Latitude (X)	Longitude (Y)	n	Prevalence RFTM (%)	RFTM-mean infection intensity (nb. of cells.g ⁻¹ of wet gill tissue)
Andernos	October 2017	44°42'31" N	1°8'11" W	46	93	4.08 x 10 ⁴ ± 6.45 x 10 ⁴
Gujan	October 2017	44°39'59" N	1°5'29" W	49	92	4.08 x 10 ³ ± 6.45 x 10 ³
IAO	October 2017	44°41'60" N	1°10'1" W	50	94	2.51 x 10 ⁴ ± 1.58 x 10 ⁵
Lanton	October 2017	44°41'31" N	1°4'48" W	50	90	1.30 x 10 ³ ± 1.85 x 10 ³
Piquey	October 2017	44°41'11" N	1°12'55" W	50	90	3.42 x 10 ⁴ ± 7.83 x 10 ⁴
Andernos	November 2018	44°42'31" N	1°8'11" W	50	90	3.18 x 10 ⁵ ± 9.09 x 10 ⁵
Gujan	November 2018	44°39'59" N	1°5'29" W	50	86	9.02 x 10 ⁴ ± 2.84 x 10 ⁵
IAO	November 2018	44°41'60" N	1°10'1" W	50	89	3.90 x 10 ⁵ ± 5.75 x 10 ⁵
Lanton	November 2018	44°41'31" N	1°4'48" W	50	74	9.07 x 10 ⁴ ± 2.57 x 10 ⁵
Piquey	November 2018	44°41'11" N	1°12'55" W	50	98	2.27 x 10 ⁵ ± 5.62 x 10 ⁵

2.1.1. Description of the two sampling campaigns

The goal of the **first sampling in 2017** was to evaluate the Perkinsosis distribution in Manila clam populations. We sampled 50 clams from each station. For *Perkinsus* cells counting, each gills clam tissues samples were dissected, weighted and incubated in RFTM (Ray’s Fluid Thioglycollate Medium) supplemented with antibiotics (penicillin 66 µg.mL⁻¹ and streptomycin 32 µg.mL⁻¹) and antimycotic (nystatin 0.04 mg.mL⁻¹). Five Manila clams per sampling site (total of 25 clams) were incubated into a tank filled with 40 L of filtered (0.2 µm) sea water at 15°C for 10 days for parasitic cultures

The goal of the **second sampling in 2018** was to evaluate the diversity and the distribution of different *Perkinsus* species occurring across the Manila clam populations. Fifty Manila clams were collected from each station. Each individual organ was independently dissected on ice and weighted. For *Perkinsus* cells counting, one gill was incubated in RFTM (Ray’s Fluid Thioglycollate Medium) supplemented with antibiotics (penicillin 66 µg.mL⁻¹ and streptomycin 32 µg.mL⁻¹) and antimycotic (nystatin 0.04 mg.mL⁻¹). The six other organs or compartments, the second gill, the digestive gland, the mantle, the adductor muscle, the foot and the remaining tissue, were fixed separately in ethanol 80 % and stored at 4°C for molecular analysis. As previously described five more Manila clams were sampled for parasitic culture (total of 25 clams). These clams were incubated into a 40 L of filtered (0.2 µm) sea water at 15 °C for 10 days.

2.1.2. Health parameters of Manila clams

All clam shells were weighted and measured to evaluate their health states and their oldness in the bay. Shell lengths were first tested for normality (Shapiro-Wilk normality test) and analysis of variance (ANOVA) was conducted before multiple comparisons of means by Tukey test.

2.1.3. Visual inspection of BMD and BMD infectious agents

Others infectious agents commonly detected in Manila clams in Arcachon Bay as the Brown Muscle Disease (BMD) suspected to be caused by viral agent (Dang *et al.*, 2009b; Pierron *et al.*, 2019) and the Brown Ring Disease (BRD) caused by *Vibrio tapetis* (Paillard *et al.*, 1994; Borrego *et al.*, 1996) were also noticed by visual diagnosis. We checked during dissection the presence of necroses of the posterior adductor muscle for the BMD (see Dang *et al.*, 2008) and the presence of a brown organic conchiolin deposit in the inner face of the valves for the BRD (see Paillard *et al.*, 2006). Prevalences (in %) for both diseases were determined for each sampling station. Their distributions were tested between each station by Fisher's exact test.

2.2. Ray's fluid thioglycollate medium (RFTM)

Body-burden assays were proceeded following the RFTM (Ray's Fluid Thioglycollate Medium) method as recommended by the O.I.E. (Ray, 1952; Choi *et al.*, 1989; O.I.E, 2019). Gill tissue samples were incubated in thioglycollate medium for five days at room temperature protected from light. This step allowed transformation of vegetative cells (trophozoites) into resting cells (hypnospores) (Ray, 1952). Then, gills were digested with 2M NaOH solution for 3 h at 60 °C preserving the *Perkinsus* hypnospores cell structure (Choi *et al.*, 1989). Recovered hypnospores were stained with a Lugol's iodine solution (4 %), and counted in a Nageotte chamber (ten lines in triplicate) under an optical microscope (Leica DM-IRB; x10 magnification). The counting results from 2017 and 2018 were expressed as the number of hypnospores per gram of wet tissue.

2.3. *Perkinsus* in vitro culture

The Manila clams kept in stabling (in 2017 and 2018 : n = 50 clams in total) were used to establish cultures by following a simplified protocol based on the original protocol of Casas *et al.* (2002). Gills were dissected and decontaminated with five successive baths of antibiotics 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. Infected tissue samples were then incubated in *Perkinsus* broth medium (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 allowing the trophozoites proliferation. Cultures were then successfully established from 17 and 20 individuals in 2017 and 2018 respectively. Species identification was carried out after two weeks of incubation at 25 °C. Briefly, parasitic cells were pelleted by centrifugation (5 000 g, 5min) and DNA was extracted following the protocol described by Itoiz *et al.* (submitted) described above.

2.4. DNA extraction of clam tissue samples

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) described by Itoiz *et al.* (submitted). Briefly, clam tissue samples were transferred in bead beating tubes containing three different sizes of beads (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). Tissues were grinded and homogenized following two bead beating cycles (45 seconds of bead beating at 6 m. s⁻¹ following by 20 seconds stop) into cooling rack of the FastPrep-24 5G (MP Biomedicals). β-mercaptoethanol (0.2 %) and proteinase K (0.1 mg.mL⁻¹) were then added in each tube and samples were incubated 30 min at 60°C. Foot tissue samples were exclusively incubated 12 hours due to its extremely hard muscled structure. Lysates were mixed in chloroform/isoamylalcohol (24:1, v/v) and emulsified prior 10 min spin at 18 000 g in a cool microcentrifuge (4 °C). This step is repeated twice for foot tissue samples. Aqueous phases were treated with RNase solution (10 mg.mL⁻¹) (Sigma-Aldrich) for 30 min at 37 °C prior DNA precipitation with cold isopropanol overnight at 4 °C. DNA was pelleted, rinsed twice with cold 70 % ethanol, dried at room temperature and resuspended in 300 µl of pure molecular

grade water (Corning). gDNA samples were quantified using the Qubit dsDNA HS assay kit (Invitrogen) and stored at -20 °C until further processing.

2.5. *Perkinsus* genetic diversity in the Arcachon bay

2.5.1. ITS rDNA genetic diversity *P. olseni* and *P. chesapeaki*

Using the *Perkinsus* genus specific primers PerkITS85 (5'-CCGCTTTGTTTGGATCCC-3') and PerkITS750 (5'-ACATCAGGCCTTCTAATGATG- 3') developed by Casas *et al.* (2002), we succeeded to amplify the partial ITS1-5.8S-ITS2 rDNA regions (~ 670 bp), as described by Itoiz *et al.* (submitted), from parasites cultures in 2017 (17 cultures on a total of 25) and 2018 (20 cultures on a total of 25). Positive PCR amplifications were then purified using the Wizard SV Gel and PCR clean-up system (Promega) as recommended by the manufacturer. Purified PCR amplicons were double-strand sequenced using PerkITS85 and PerkITS750 primers by Eurofins Genomics company. The species identification was confirmed by firstly a blast against NCBI non-redundant (nr) database (accessed December 2017 and December 2018). Good-quality sequences were included into a multiple sequence alignment of available *Perkinsus* ITS rDNA sequences.

2.5.2. Phylogenetic analysis of *Perkinsus* sequences from Arcachon bay

Genbank sequences of ITS1-5.8S-ITS2 region from seven *Perkinsus* species (*P. olseni*, *P. chesapeaki*, *P. marinus*, *P. honshuensis*, *P. beihaiensis*, *P. mediterraneus* and *P. qugwadi*) were aligned with *Perkinsus* amplicon sequences from the Arcachon bay using Clustal W implemented in MEGA X software v. 3.2 (Kumar *et al.*, 2018) (Table S1). A total of 14 sequences of *Perkinsus* cultures isolated from Manila clams from the Arcachon bay were retained for phylogenetic analysis. These sequences were assembled into a multiple sequence alignment with 55 *Perkinsus* spp. sequences recovered from NCBI non-redundant (nr) database (accessed October 2020; see Figure S1 and Table S1). Sequences were aligned using the MAFFT (v. 7.402) using iterative refinement method L-INS-I (Kato *et al.*, 2005) available on CIPRES online platform (Miller *et al.*, 2010). Alignment was subsequently edited manually and masked with Seaview 5.0.4 (Gouy *et al.*, 2010). The alignment was checked and manually improved in Seaview, resulting in a matrix of 69 sequences and 472 alignment positions with *Perkinsus qugwadi* sequences (AB973172, AB973173, AB973174, AB973175, AF151528) used as outgroup.

The best fitting nucleotide substitution model for the ITS alignment was determined using jModelTest2 v. 2.1.6 (Guindon and Gascuel, 2003; Darriba *et al.*, 2012). A GTR+ Γ model was then selected with α parameter for the Γ distribution of 0.99 with eight discrete rate categories. Neighbour joining (NJ) phylogeny was built using LogDet distance implemented in Seaview v.3.0 software. Bootstrap support values were estimated using **1)** LogDet distance analysis with 1 000 pseudoreplicates using a NJ search method (available through Seaview v4.2); **2)** Maximum likelihood (ML) using MEGA X (Nei and Kumar, 2000) with 1 000 pseudoreplicates; and **3)** Bayesian posterior probabilities were calculated using the following parameters on the software MrBayes v3.2.6 implemented in Geneious software, model GTR+ Γ (lset, nst=6 rates=gamma) was selected. The chains were run for 2 000 000 generations with two replicate tree searches both with 4 MCMC chains with heat parameter of 2. Trees were sampled every 250 generations. In both analyses, the MCMCMC searches had converged within the first 25% of the generations sampled, as such the first ¼ of the search results were discarded (as the burnin). The consensus topologies and posterior probabilities of each node were then calculated from the remaining trees.

All Perkinsus ITS rDNA sequences generated as a part of this study will be deposited in GenBank. The mask and unmask alignment will be available under the Seaview mase format.

2.6. Real-time qPCR duplex diagnostic assay for *P. olseni* and *P. chesapeaki*

Distribution and infection intensity were investigated using the duplex qPCR methodology developed in Itoiz *et al.* submitted (Table 2). *P. olseni* and *P. chesapeaki* plasmidic-standards from 2.5×10^6 to 2.5×10^1 total copy number were used to calculate infection intensity. This standard-plasmidic material were already used in Itoiz *et al.* (submitted) allowing identical qPCR parameters in this study (*P. olseni*: $y = -3.33x + 39.49$, efficiency (E) = 99.8 %, limit of detection (LOD) = 2.5×10^1 total copies; *P. chesapeaki*: $y = -3.38x + 40.29$, E = 97.8%, LOD = 2.5×10^1 total copies).

Briefly, each gDNA organ was diluted following specific concentrations recommended in Itoiz *et al.* (submitted) to avoid PCR inhibitors. Gill and digestive gland tissue samples were diluted to $20 \text{ ng} \cdot \mu\text{L}^{-1}$; adductor muscle and foot to $5 \text{ ng} \cdot \mu\text{L}^{-1}$; and, mantle and remaining tissue to $2 \text{ ng} \cdot \mu\text{L}^{-1}$. Real-time PCR was performed using the LightCycler 480 II (Roche) thermocycler. All qPCR assays were performed following recommendation of the LightCycler 480 Probes Master

(Roche) kit. Primers and probes were respectively diluted in pure water at 50 μ M and 2 μ M 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 MgCl₂. The reaction volume for duplex qPCR consisted of 1X LightCycler 480 Probes Master premixed, 0.2 μ M of primer P_{olsITS2-F}, 0.2 μ M of P_{olsITS2-R}, 0.2 μ M of P_{chesITS2-F}, 0.2 μ M of P_{chesITS2-R}, 0.5 μ M of P_{olsITS2-probe} (FAM), 0.5 μ M of P_{chesITS2-probe} (LC640), and 5 μ L of DNA sample with adjusted concentration. The reaction volume for simplex qPCR consisted of 1X LightCycler 480 Probes Master premixed, 0.2 μ M of primer P_{olsITS2-F} or P_{chesITS2-F}, 0.2 μ M of P_{olsITS2-R} or P_{chesITS2-R}, 0.5 μ M of P_{olsITS2-probe} or P_{chesITS2-probe}, 5 μ L of DNA sample with adjusted concentration. The thermal cycling conditions were as followed: 5 min of pre-incubation at 95 °C followed by 45 cycles of amplification containing 10 s of denaturation step at 95°C and 20 s of annealing step 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). PCR assay finally terminated by a last cooling period of 2 min at 40 °C. In each qPCR run, diluted gDNA samples, plasmidic-standards and two negative controls (non-infected Manila clam DNA and pure molecular grade water) were run. Ct values triplicates were averaged for downstream statistical analysis. If one or two triplicates were anomalous, sample was reprocessed to discard a potential manipulation error.

Table 2. Primers and probes sequences specific to *P. olseni* and *P. chesapeaki* ITS2 sequence region of nuclear ribosomal DNA. Excitation wavelengths of FAM fluorochrome: 465-510 nm and LC640 fluorochrome: 618-660 nm.

Target	Primer /probe	Sequence (5'->3')	T _m (°C)	Amplicon size
ITS 2 <i>P. olseni</i>	P _{olsITS2-F}	CACCACAACACAGTCGGAC	58,8	76 bp
	P _{olsITS2-R}	CGTATTGTAGCCCCCTCCGA	58,8	
	P _{olsITS2-probe}	FAM-GACACTCACAGGCGGGTCC-[BHQ2]	65,7	
ITS 2 <i>P. chesapeaki</i>	P _{chesITS2-F}	GGAACACGGAATCAACCACA	57,3	87 bp
	P _{chesITS2-R}	ACTGTAGCCCCCTTCGCAAG	58,8	
	P _{chesITS2-probe}	LC640-AGTCGAGTTCGCGAGCATCCAA-[BHQ2]	63,3	

2.7. Statistical analysis of prevalences and infection intensities

2.7.1. Comparison of RFTM and qPCR methodologies on gill tissue samples from 2017 and 2018

To confirm congruence between RFTM and qPCR methodologies, gill tissue samples were chosen to calculate indicative parameters like prevalence, concordance and infection intensity following the recommendation as a proxy for *Perkinsus* spp. diagnosis by O.I.E. (O.I.E, 2019). Briefly, *Perkinsus* prevalences (% proportion of *infected* individuals to the total number of hosts sampled) were plotted for both methods and the single- or co-infection was represented when possible. Two parameters, the concordance and the discordance, adapted from Langton *et al.* (2002), were calculated. The concordance, calculated by counting paired of positive samples and paired of negative samples, is the percentage of chance that an identical sample analysed by two different methodologies will be given the same result. The discordance, calculated by counting the unpaired samples (i.e. positive in RFTM and negative in qPCR assay, and vice versa), is the percentage of chance that an identical sample analysed by two different methodologies will be given different results. Finally, the relationship between both methods was tested using a linear model and the Spearman correlation coefficient on infection intensities established by qPCR and RFTM from paired-positive individuals.

The global prevalence and infection intensities of the *Perkinsus* genus in 2017 and 2018 were calculated on gill samples using standard RFTM methodology. Means of infection intensity (nb of hypnospores. g⁻¹ of wet gill) measured by the two methods were compared to each other by a non-parametric Kruskal-Wallis test followed by Dunn post-hoc test.

2.7.2. Distribution and infection intensity of two *Perkinsus* species in host tissue samples

Infection intensity, prevalence and distribution of *P. olseni* and *P. chesapeaki* were estimated using the duplex qPCR methodology across six Manila clam organs: gills, digestive gland, adductor muscle, foot, mantle and the remaining tissue. Prevalences in each station were compared by Fisher's exact test, adapted for small samples. Mean of infection intensity was evaluated in each station for each type of infection (*P. olseni* single-infection, *P. chesapeaki* single-infection, co-infection) and were compared by a *pairwise Wilcoxon rank sum test*.

The distribution of single- and co-infected individuals in the Arcachon bay was investigated using multivariate analysis on *P. olseni* and *P. chesapeakei* infection intensities in each organ. A principal component analysis (PCA) was performed using FactomineR package on R (Lê *et al.*, 2008). Extracted coordinates (axis 1; 2) of each individual were checked for significant differences among stations by non-parametric Kruskal-Wallis test in combination with multiple pairwise comparison using Dunn post-hoc test.

Repartition of both *Perkinsus* species across co-infected host organs (gills, digestive gland, adductor muscle, foot, mantle and the remaining tissue) was detailed in a visual matrix table. Global infection intensities (copy number.g⁻¹ of wet tissue) for *P. olseni* and *P. chesapeakei* were log-transformed and classified in four levels and represented following colour gradient. Infection intensities data follows a Gaussian distribution. Thus, the boundaries of the four categories were delimited by: minimum, 1st quartile, median, 3rd quartile and maximum values of infection intensity. Mean occupation (%) of *P. olseni* and *P. chesapeakei* was represented for each type of co-infected organ sample from each station. Finally, *P. olseni* and *P. chesapeakei* log-transformed infection intensities from co-infected organs were plotted to investigate potential negative or positive influence of one species on the other and vice versa. Based on a reduced dataset restricted to co-infected organ samples, the relationship was tested using a linear model and Spearman correlation coefficient.

3. Results

3.1. Confirmation of co-infection and qPCR duplex methodology

3.1.1. Diversity of *Perkinsus* species in the Arcachon bay

To investigate the genetic diversity of the *Perkinsus* species in the Arcachon bay, we sequenced and analysed the ITS1-5.8S-ITS2 region of 14 parasitic cultures isolated from *R. philippinarum* (Figure S1; Table S1). All phylogenies recovered a strongly supported backbone as previously described in phylogenies of *Perkinsus* species (Kotob *et al.*, 1999; Park *et al.*, 2005; Elandaloussi *et al.*, 2009). The ITS1-5.8-ITS2 sequences recovered here branch either within *P. olseni* clade with high bootstrap values (100 %/ 99% /1) or with *P. chesapeakei* clade with moderate bootstrap values (75 %/63 % /0.7). These phylogenies provided a strong support for the phylogenetic affiliation of Arcachon sequences to *P. olseni* or *P. chesapeakei* clades. Total

of nine *P. olseni* sequences (2017: n= 6; 2018: n = 3) and five *P. chesapeaki* sequences (2017: n= 1; 2018: n= 4) from cultures were selected on the criteria of good sequencing quality and length.

Phylogenetic analysis demonstrated that *P. olseni* forms one clade with strong bootstrap value (100%/99%/1) displaying more than 98 % of sequence identity. This clade encompassed our nine cultures sequences isolated from the Arcachon bay and 12 *P. olseni* sequences from Korea (KX514117, KX514123, KX514103), Japan (DQ51614, LC431768), India (MG733365, MG73337), Spain (KP764681, KP764683, FJ481986, EU293848) and France (JQ669642) available on Genbank (Figure S1; Table S1). The variability in sequence nucleotide was of 0.7 ± 0.3 for *P. olseni* showing that this marker was not sufficient to determine geographical origin of *P. olseni* sequences. Conversely, *P. chesapeaki* cluster shared 96 % of sequence identity with 9.1 ± 1.9 variables sites per sequence. This cluster was composed of two strongly supported clades formed by 25 Australian (KM983419, KM983409, KM983418, KM983413, KM983414, KM983417, KM983416, KM983411) and USA (AF440466, AF102171, AY876307, AY876305, AY876303, AY876302, AY876304, AY876306, EU919484, EU919470, AF252288, AF440464, AF440468, AY305326, AF091541, KF314812) sequences sharing 98 % of sequence identity and seven European sequences (Spain: KP764686, KP764685, KP764684; France: JQ669646, JQ669647, JQ669649, JQ669648) sharing 99 % of sequence identity. Two cultures sequences from 2018 formed a third clade supported by a weak bootstrap value composed of AR18-4P3, AR18-1P11 and *P. chesapeaki* sequence JQ669648 from Arzul *et al.* 2012. This weak support value within the *P. chesapeaki* clade may correspond either to the low number of *P. chesapeaki* sequences from Europe available in GenBank or to some nucleotidic indetermination that were still present in the nucleotide sequences.

Low number of sequences retrieved for *P. chesapeaki* were explained by low quality when sequenced and too short sequences. Co-occurrence of *P. olseni* and *P. chesapeaki* in the same culture or tissue could interfere in PCR amplification when using ITS *Perkinsus* specific primers. Implementation of poor-quality sequences in phylogenetic analysis could increase artificially the diversity and weaken topology of the tree.

3.1.2. Comparison of *Perkinsus* spp. infection between the RFTM and the duplex qPCR assay on gills tissue samples

The duplex qPCR assay, developed by Itoiz *et al.* (submitted), allowed the simultaneous detection and quantification of both parasitic species, *P. olseni* and *P. chesapeaki* in Manila clams. We firstly evaluated the concordance and correlation between RFTM and duplex qPCR methodology on gill tissue samples across all sampling stations. To guarantee a good representation of the qPCR detection, only Ct values within the standard range from 2.5×10^1 to 2.5×10^6 total copies number are considered as positive as recommended by Itoiz *et al.* (submitted). All Ct values out of range were discarded because considered as unquantifiable.

Using the O.I.E recommended RFTM and qPCR assays, global prevalences of *Perkinsus* species, based on gill tissue diagnosis, did not show any significant differences across sampling stations (Chi-squared test: $X^2 = 4.14$, $df = 4$, $p\text{-value} > 0.05$). (Figure S2). Prevalences of *Perkinsus* spp. determined by RFTM were estimated between 74% to 98% in Lanton and Piquey stations respectively. Using the molecular method, prevalences were estimated between 62% to 90% respectively in Gujan and IAO. Thus, the concordance across all stations varied between 76 % and 86 % (Table S2). Mean concordance between the RFTM and the duplex qPCR methodology was $81.6 \pm 5,2$ %. In Gujan and Andernos, the low concordance value could be explained by several samples positive in qPCR but situated out of Ct values range (below 2.1×10^1 total copies). Thus, these samples were considered as unquantifiable and have been discarded from the analysis.

In Itoiz *et al.* (submitted), a linear-relationship has been established between RFTM and qPCR results obtained on gill tissue samples from Lanton. Here, we investigated if this linear-relationship was coherent for all gill tissue samples from different stations in the bay. A linear regression was determined between for the qPCR-infection intensity and the RFTM-infection intensity ($y=0.56x+4.34$, y : log of qPCR-infection intensity in copy number.g of wet gill⁻¹; x : log of RFTM-infection intensity in nb of cells.g of wet gill⁻¹; adjusted-R²=0.41; n=178) (Figure S3). A significant Pearson's coefficient was also obtained between the qPCR-infection intensity and the RFTM-infection intensity ($r=0.64$, $p\text{-value} < 0.001$) showing a positive correlation. The intercept of this linear relationship was not equal to zero which introduce an overestimation of infection intensities by the qPCR assay.

3.1.3. New insights of *Perkinsus* infections in gills using molecular detection

The molecular method allowed, for the first time, the estimation of the prevalence and infection intensity at species level avoiding culture bias (Rios *et al.*, 2020; Marquis *et al.*, 2020). These data were important to evaluate parasitic associations occurring in host's gills, considered as the portal of entry of *Perkinsus* infection. Here, we showed that *P. olseni* single-infection prevalences were estimated between 58 % (29/50 clams, positive clams/total of clams tested) to 84% (42/50 clams) in Piquey and Lanton respectively. Conversely, no *P. chesapeaki* single-infection was detected in gills samples in these two sampling stations. Prevalences of co-infection between *P. olseni* and *P. chesapeaki* were estimated from 6 % (3/50 clams) in Lanton and IAO to 24 % (12/50 clams) in Piquey. No co-infection was detected in gill samples from Gujan and Andernos. However, considering the low number of co-infected hosts (n=3) in Lanton and IAO, these results might be considered with cautious.

3.2. Prevalence and infection intensity of *P. olseni* and *P. chesapeaki* at local scale: single- and co-infections.

3.2.1. Diagnostic of the Manila clam whole body

Global screenshot of single- and co-infection across the Arcachon bay was assessed by analysing the whole body of Manila clams with duplex qPCR method. Prevalences and infection intensities were estimated on the whole body of Manila clams. In this study, a clam was identified as “co-infected” when *P. olseni* and *P. chesapeaki* infect at least one of the six organs analysed.

The global prevalences of *Perkinsus* spp. ranged from 82 % for Lanton, Andernos and Gujan, 90 % for Piquey to 96 % for IAO (Figure 1A). Prevalences of *P. olseni*-single-infection were detected in every station from 46 % in Gujan (n= 23/50 clams) to 82 % (n= 41/50 clams) in Andernos. Prevalences of the co-infection were detected in all stations except Andernos from 12 % (n= 6/50 clams) in Gujan to 36 % (n=18/50 clams) in Piquey. Surprisingly, prevalences of *P. chesapeaki* single-infection were barely detected with 2 % (= 1/50 clams) in IAO and 14 % (= 7/50 clams) in Gujan.

IAO and Gujan stations showed a similar infection type pattern (Fisher's exact test: p-value > 0.05, group a; Figure 4) with both high prevalence of co-infection. Conversely, Andernos, Gujan and Lanton showed a different infection type pattern (Fisher's exact test: p-value > 0.05,

noticed by b, c, d; Figure 4). More specifically, Gujan and Andernos showed peculiar infection type patterns with high prevalence of *P. chesapeaki*-single infection for Gujan (14%, n=7/50 clams) and absence of detection *P. chesapeaki* in Manila clam samples for Andernos. Infection intensities in the whole body showed differences solely in *P. olseni*-single infections (Figure 1B). Indeed, IAO had a higher level of infection with 8.5 log-nb. of copies. g⁻¹ of wet tissue compared to the four other stations reporting 7.2 log-nb. of copies. g⁻¹ of wet tissue in Gujan to 8.4 log-nb. of copies. g⁻¹ of wet tissue in Andernos (Wilcoxon rank sum test: p-value < 0.05; groups a and b; Figure 1B). For co-infected hosts, no differences of infection intensities between stations were observed (Wilcoxon rank sum test: p-value > 0.05; groups a and b; Figure 1B). Solely *P. chesapeaki*-single infection reported very low infection intensity with a mean of 4.8 log-nb. of copies. g⁻¹ of wet tissue (i.e. 2.2 x 10⁵ copy number.g⁻¹ of wet tissue) in Gujan. Other emerging diseases were visually inspected during clams' dissection and only two clams within the co-infected clam populations showed a brown organic conchiolin deposit classically identified as BMD phenotype (clam n°40 and n°25 in Gujan and Lanton respectively). No BRD phenotype was observed in the co-infected clams sampled.

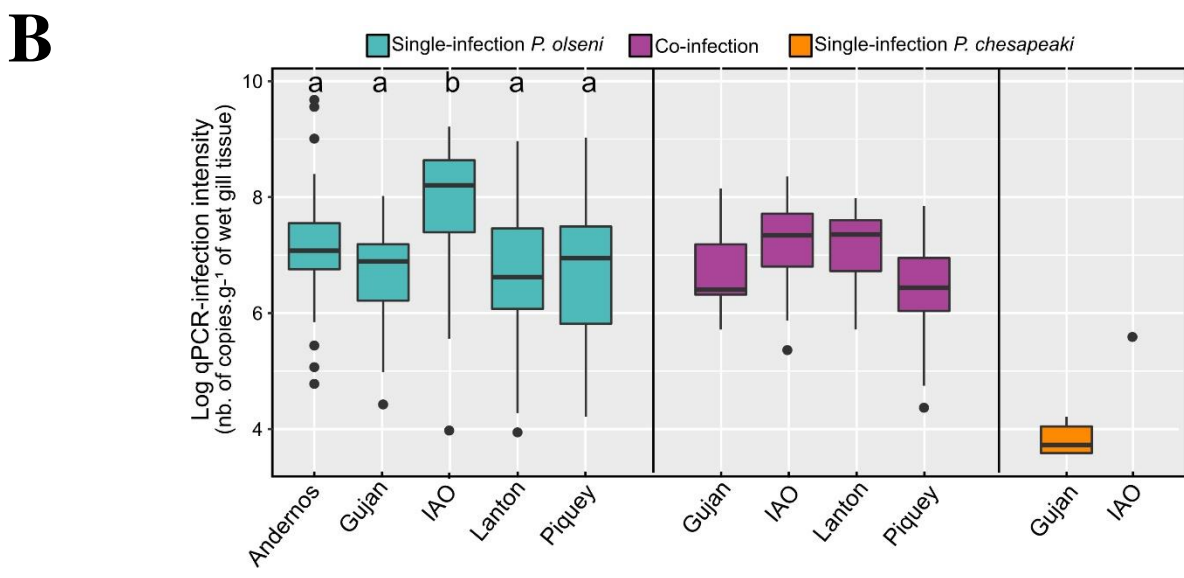
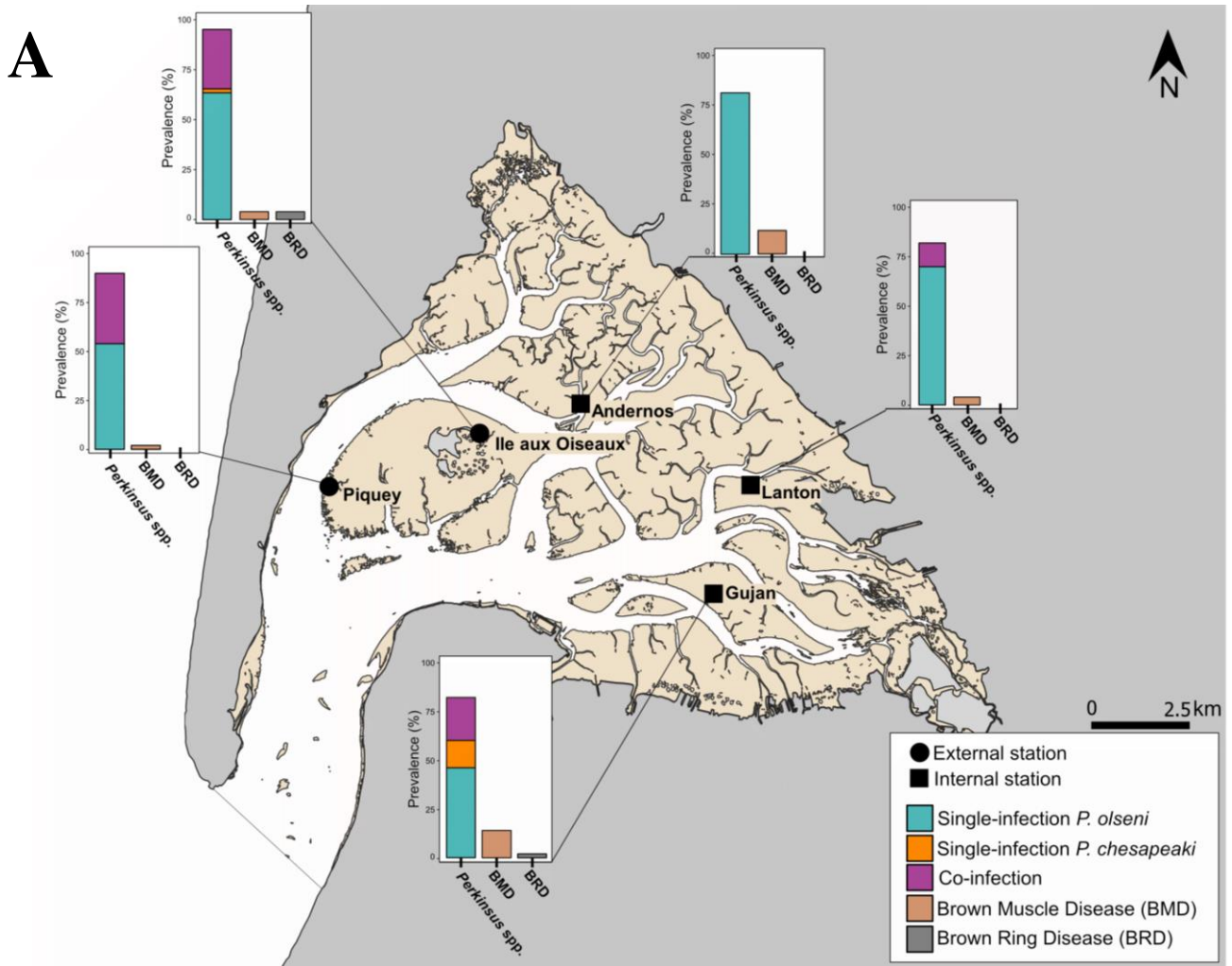


Figure 1. Distribution of infection and co-infection in whole clam body from five stations sampled in November 2018 in the Arcachon bay. **A.** Prevalences of *P. olseni*, *P. chesapeaki* (co-)infections determined by duplex qPCR with prevalences of Brown muscle disease (BMD) and Brown ring disease (BRD). Each prevalence was determined on a total of 50 clams. **B.** Log-qPCR infection intensities estimated on the whole body clam are represented for each type of infection: *P. olseni* single-infection, *P. chesapeaki* single-infection and co-infection. Differences are estimated by a pairwise Wilcoxon rank sum test. Differences between each parameter are represented by a, b and c.

3.2.2. Local patterns of *Perkinsus* spp. infection

The results of the principal component analysis (PCA) performed on infection intensities of *Perkinsus* species are presented in Figure 2. The Axis 1, representing 30 % of the total variance, was mainly explained by *P. olseni* infection intensities within the different organs, with contribution ranging from 12% for foot to 21 % for adductor muscle (Figure 2A). The Axis 2, representing 11.1 % of the total variance, was mainly explained by shell length (mm) with 37 % of contribution (Figure 2A). Ellipses of IAO and Gujan are well separated along the Axis 1 whereas those of Andernos, Lanton and Piquey overlap. Indeed, IAO station was characterised by higher *P. olseni* infection intensity contrary to Andernos (Figure 2B; Dunn post-hoc test: p-value <0.01). Conversely, ellipse of Piquey station was well separated along the Axis 2 (Figure 2A). Individual clams from Piquey station were characterised by high shell length (35.5 ± 2.7 mm; Tukey multiple comparison means test: p-value <0.001) and occurrence of *P. chesapeaki* in gills (Figure 2B; Figure 3). Other stations were characterised by smaller individual clam shell lengths (IAO 28.2 ± 2.1 mm and Lanton 29.3 ± 2.3 mm compared to Andernos 31.9 ± 2.6 mm and Gujan 32.4 ± 2.6 mm; Tukey multiple comparison means test: p-value <0.001) and *P. chesapeaki* mainly localised in the adductor muscle (Figure 2B & 3).

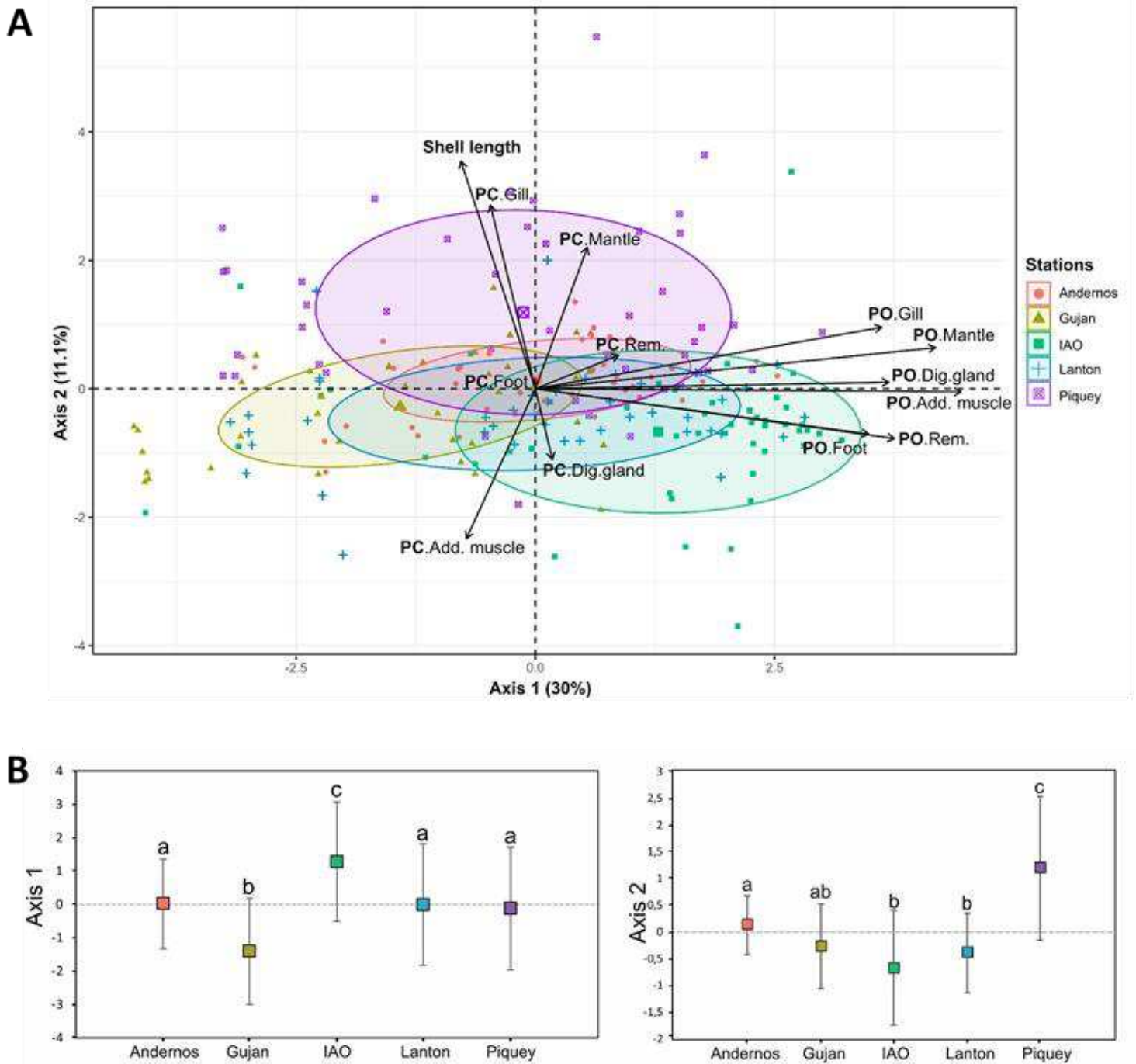


Figure 2. Principal component analysis (PCA) of *P. olseni* and *P. chesapeaki* infection intensities in different Manila clam organs from Arcachon bay in November 2018. The first axis explains 30 % of total variance of infection intensities while axis 2 explains 11.1 %. **A.** PCA including 250 individuals coloured by station. Ellipses included at least 50% of the individuals in a station. Variables implied in the PCA are the shell length and infection intensities specified by PO for *P. olseni* and PC for *P. chesapeaki* followed by the type of organ. Dig. gland: digestive gland, Add. muscle: adductor muscle, Rem. tissue remaining tissue. **B.** Individual values grouped by station on the Axis 1 and 2 of the PCA represented by infection intensities and shell length. Differences between stations were indicated by letters a, b and c according to multiple pairwise comparison of Dunn post-hoc test (p -value < 0.05).

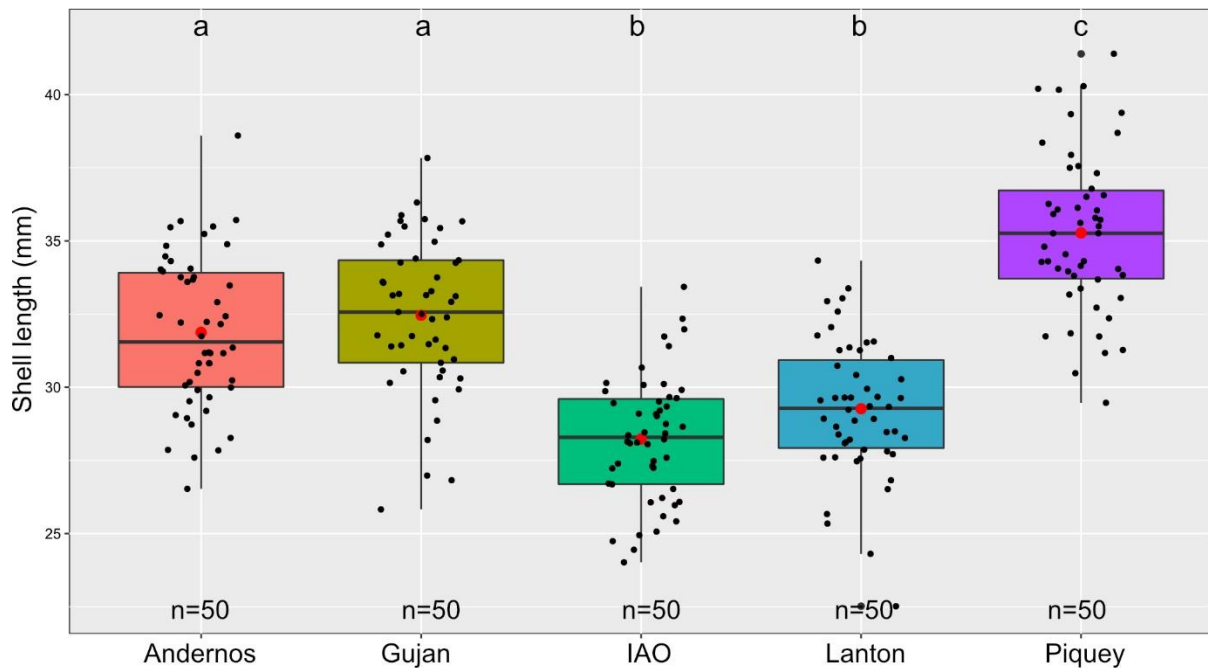


Figure 3. Shell length of Manila clams sampled Arcachon bay in 2018. Stations were compared each other. Shell length variable is normally distributed (Shapiro-Wilk normality test: $W = 0.99$, $p\text{-value} = 0.2$). Analysis of variance (ANOVA) demonstrates significant difference between station ($p\text{-value} < 0.001$). A Tukey multiple comparisons of means test (95% family-wise confidence level) were realised to determine significant difference between stations depending on shell length of Manila clam. Differences are represented by a, b and c.

3.3. Patterns of the co-infection

Distribution of *P. olseni* and *P. chesapeaki* in each Manila clam organs was investigated to evaluate more accurately potential patterns in co-infected clams. A clam was considered as co-infected when both parasites are detected in at least one of the six organs ($n = 50$ co-infected individuals dispatched in four stations). The molecular method allowed detection of parasitic organisation at individual scale while avoiding the PCR inhibitory effects specific to each organs.

P. olseni appeared to be distributed in all organs tested with high infection intensity reaching up 9.1 log of copy number.g⁻¹ of wet tissue (Figure 1B & 4). In co-infected hosts, *P. olseni* reached maximum of 8.3 log of copy number.g⁻¹ of wet tissue (Figure 4). Conversely, *P. chesapeaki* was more heterogeneously distributed with weak infection intensity up to 6.9 log of copies. g⁻¹. Our results highlighted three different profiles between four sampling stations: **1)** co-infection was often detected in the gill tissue with medium infection intensity for *P. chesapeaki* like in Piquey (between 5.2 to 5.6 log of copy number.g⁻¹ of wet tissue, $n = 11/18$

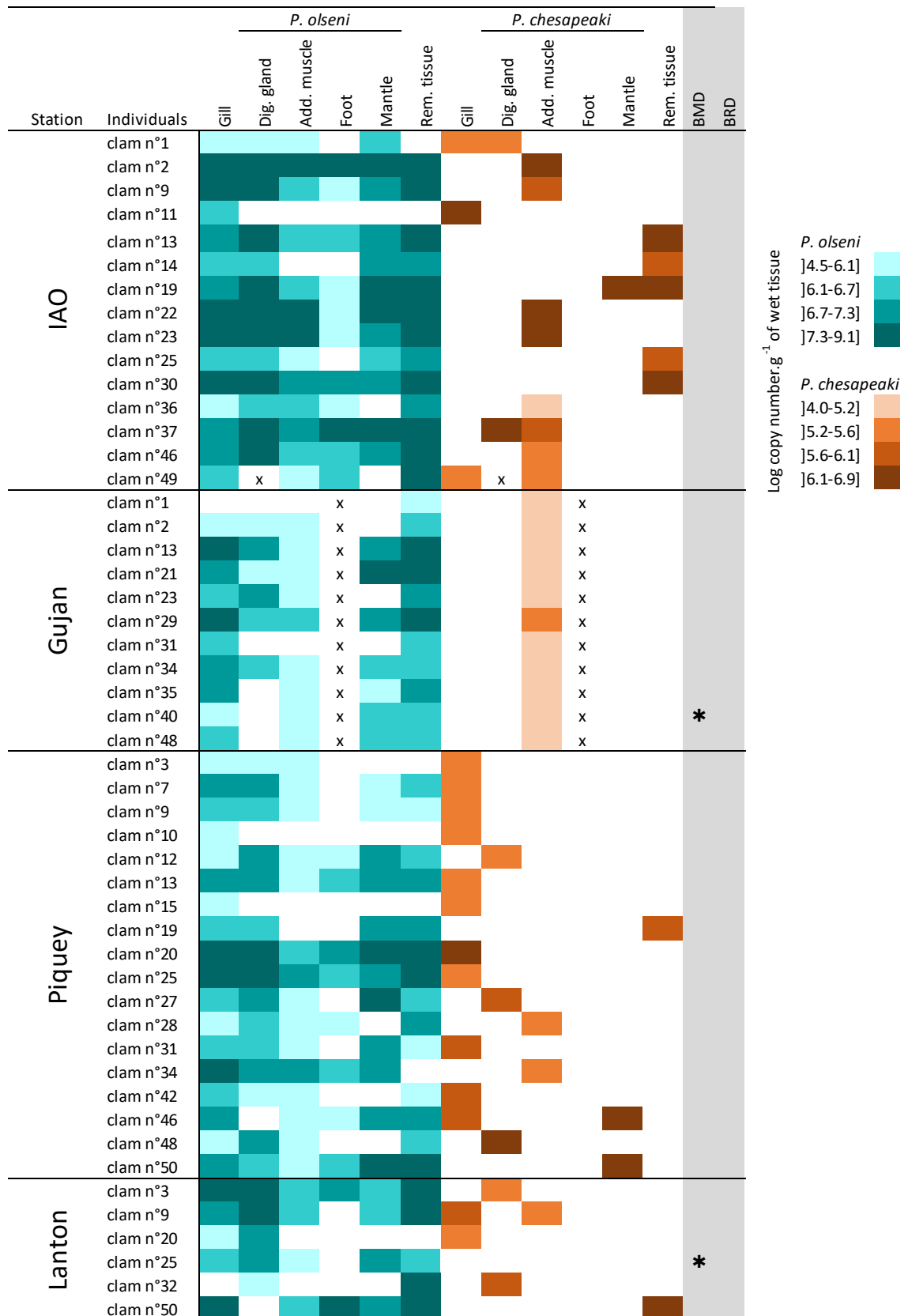


Figure 4. Matrix of co-infected individuals determined by qPCR duplex assay. The *P. olseni* repartition is represented in blue and the *P. chesapeaki* repartition in orange. The RFTM infection intensities are represented in grey. Four levels of infection intensities were determined. x: missing value. Dig. gland: Digestive gland; Add. muscle: Adductor muscle; Rem. tissue: Remaining tissue. *: occurrence of Brown Muscle Disease (BMD) or Brown Ring Disease (BRD).

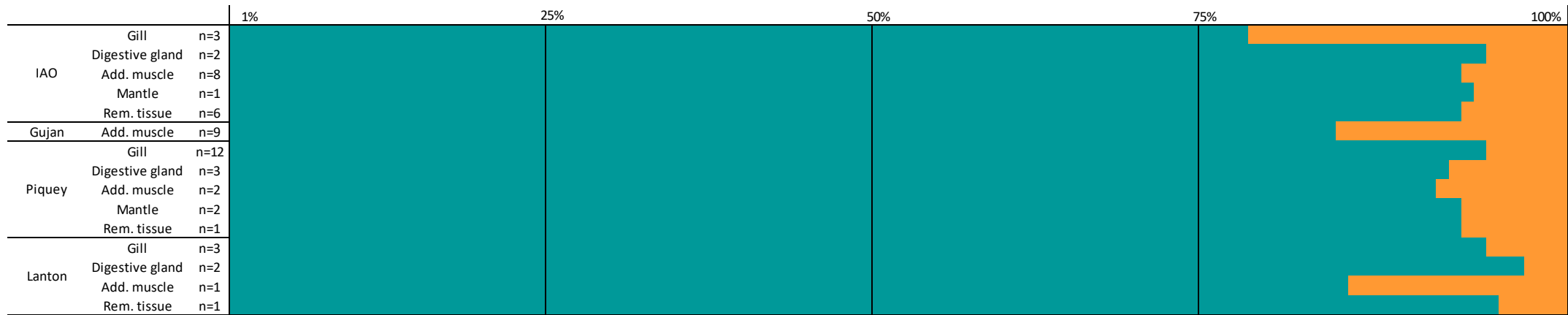
clams); **2)** the co-infection was detected in the adductor muscle with moderate infection intensity in IAO (5.6 to 6.9 log of copy number.g⁻¹ of wet tissue, n= 8/15 clams) or with low infection intensity in Gujan (4.0 to 5.6 log of copy number.g⁻¹ of wet tissue, n= 11/11 clams). In IAO, 53% of co-infected Manila clams (n=8/15 clams) were characterised by co-infection in the adductor muscle contrary to Gujan where it was detected exclusively in the adductor muscle; and **3)** co-infection were sporadically detected in digestive gland, mantle or remaining tissue dispatched across stations.

Proportion of *P. olseni* and *P. chesapeaki* in different compartments (gill, digestive gland, adductor muscle, mantle and remaining tissue) were assessed based on mean infection intensity (nb of copy number. g⁻¹ of wet tissue) to determine their respective use of the space resource (Figure 5; Table S3). We showed that *P. olseni* dominated all co-infected organs with a mean occupation of *P. olseni* of 92 ± 6 %. For *P. chesapeaki* the mean occupation is solely estimated at 8 ± 6 %. In gill tissues, *P. olseni* weakest proportion was detected mainly in IAO (n=3) with 77% of occupation while it was detected up to 96 % for Piquey (n=12) and Lanton (n=3). In adductor muscle, the *P. olseni* lowest proportions were detected in Gujan (n=9) and Lanton (n=1) with 84 % and 85 % respectively. In every others organ (digestive gland, mantle and remaining tissue), *P. olseni* occupation was higher than 90 % (n=18).

While in Gujan *P. chesapeaki* was only detected in adductor muscle (Figure 4), weights of foot samples were missing. However, no co-infection has been detected in foot samples of other individuals from other stations, so we can assume that this trend is similar for *P. chesapeaki* in the Gujan station.

To understand the co-infection distribution, infection intensities of *P. olseni* and *P. chesapeaki* from co-infected organ samples were plotted. We identified linear regression connecting both parasitic species ($y=0.51x+2.2$, adjusted-R²=0.64, n=54 of co-infected organs). A significant Pearson's coefficient showed a strong positive correlation between *P. olseni* and *P. chesapeaki* infection intensities ($r=0.81$, p-value < 0.001). Mantle (n=3) and remaining tissue (n=8) samples showed the highest infection intensities of *P. olseni* (> 6.7 log of copy number. g⁻¹ of wet tissue) and *P. chesapeaki* (> 5.6 log of copy number. g⁻¹ of wet tissue). Gill, digestive gland and adductor muscle samples did not form specific aggregation following infection level. However, low values of *P. olseni* (< 6.1 log of copy number. g⁻¹ of wet tissue) and *P. chesapeaki* (< 5.2 log of copy number. g⁻¹ of wet tissue) were mainly represented in adductor muscle (n=8) (Figure 6). This relationship exposed that the co-occurrence of both species in a same organ, concerning

Figure 5. Mean proportions of *P. olseni* and *P. chesapeaki* in common co-infected organ. Proportions are represented by the percentage of the mean of each parasitic species related to the total of copies of parasites in the organ. n: the number of common organ infected by *P. olseni* and *P. chesapeaki*. *P. olseni* is represented in blue and *P. chesapeaki* in orange. Means and standard-deviation are detailed in Table S4. Add. muscle: Adductor muscle; Rem. tissue: Remaining tissue. Means and standard deviations are detailed in Table S3.



96 % of co-infected hosts (n=48/50 co-infected clam), happened in a restricted range of infection intensity from 4.5 and 6.9 log of copy number. g⁻¹ of wet tissue. Above this limit range, only *P. olseni* mono-infection and co-infection in separate organs (n=2) were noticed.

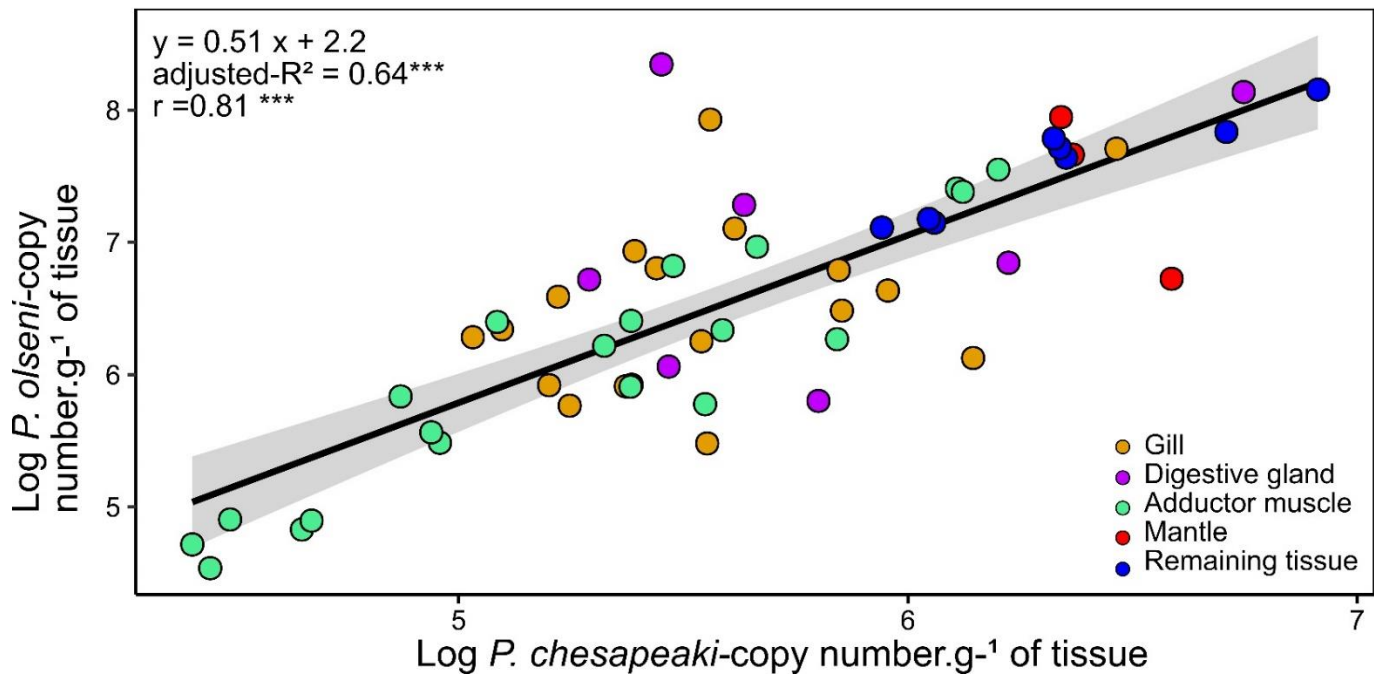


Figure 6. Linear regression between *P. olseni* and *P. chesapeaki* infection intensities (copy number.g⁻¹ of wet tissue) from co-infected organ samples (n=54). The linear relationship corresponds to the equation model: $y = 0.51 x + 2.2$ and explains 64% (adjusted-R²) of the variability. The relationship Organs (gill, digestive gland, adductor muscle, mantle and remaining tissue) were represented with coloured circles. r: Pearson's coefficient. '***' p-value < 0.001.

4. Discussion

The development of the duplex qPCR methodology allows the quantification of infection intensity and prevalence of both parasitic species *P. olseni* and *P. chesapeaki* across Manila clam population from the Arcachon bay as described by Itoiz *et al.* (submitted) (Figure S1 & S2). The concordance parameter ($81,6 \pm 5,2$ %) and the linear regression ($y=0.61x+4.34$, $R^2=0.41$; Spearman correlation coefficient: $r = 0.64$, p -value <0.001) strengthens the reliability of this molecular method to assess prevalences of *Perkinsus* species from *in situ* samples even if it overestimates low infection intensities. Here we showed that, for the first time, in the Arcachon bay, the distribution of *P. olseni* single-infection, *P. chesapeaki* single-infection and co-infection simultaneously within Manila clam population using culture-independent methodology.

4.1. Single-infection and co-infection in the Arcachon bay

4.1.1. At local scale

The monitoring of single-infections and co-infections highlights a better understanding of Perkinsosis in the Arcachon bay. In this study, stations are mainly differentiated following *P. olseni* infection intensities with: low values in Gujan, high values in IAO and moderate values in Andernos, Lanton and Piquey. Samples with high infection intensity are mainly represented in the external station IAO, while low-moderate intensities are mainly found in inner stations. The only exception is for Piquey, which is characterised by oceanic conditions but harbour moderate infection intensity as determined for Lanton and Andernos. This distribution could be explained by environmental factors, such as lower salinity and muddy sediment in inner stations due to stronger influence of continental freshwater input or higher salinity and medium sand composition in external stations (Dang *et al.*, 2010b; Binias *et al.*, 2014a). Indeed, the Arcachon bay is a peculiar ecosystem where marine and continental water inputs mix, resulting in temperature and salinity gradients (Plus *et al.*, 2006). In 2013, Dang *et al.* demonstrated lower infection intensity close to the Leyre river which is coherent with observations made on low-moderate infection intensities in Gujan and Lanton (7.2 ± 7.4 and 7.8 ± 8.3 log of copy number.g⁻¹ of wet tissue). Conversely, higher infection and prevalence of *P. olseni* should be found in highest salinity (Park and Choi, 2001; Dang *et al.*, 2010b) considering that *P. olseni*

optimal salinity ranged from 25 to 35 psu (Auzoux-Bordenave *et al.*, 1996). Oceanic stations are characterised by higher salinity compared to inner stations (reviewed in Dang *et al.*, 2010b; Binias *et al.*, 2014a) which may explain such dichotomy in our observations except for Piquey. Piquey is a peculiar station influenced by oceanic current but harbour lower infection intensities compared to IAO. This exception could be explained by its proximity to the mouth of the bay; Manila clams might have better accessibility to fresh input of oceanic phytoplankton and benefit to a more stable salinity across the year (Dang *et al.*, 2009a, 2010a,b). The gradient of infection demonstrated here is less contrasted as previously reported by Dang *et al.* in 2013. Moreover, there is no clear distribution of co-infection between stations except for Piquey. Significant occurrences of *P. chesapeaki* in gills appeared to depend on clam's shell size suggesting a stronger primary infection of the parasite by higher clearance activity. Despite this exception, *P. chesapeaki* appears here to be a secondary actor in global *Perkinsus* infections in Manila clams.

In this study, prevalences of the Brown muscle disease (BMD), caused by a virus and Brown ring disease (BRD), caused by a bacteria are quite low compared to previous monitoring in the bay (Lassalle *et al.*, 2007; Dang *et al.*, 2008; Binias *et al.*, 2014a,b). However, our results showed here that the two pathogenic agents, which can be responsible to Manila clam mortality (Dang *et al.*, 2008), are not correlated to Perkinsosis distribution and vice versa.

4.1.2. At individual scale

Considering the whole Manila clam body, single-infection of *P. olseni* is dominant across all stations and represented all cases of infection in Andernos. Co-infection was regularly detected across Gujan, IAO, Lanton and Piquey from 12 % to 36%. Conversely, *P. chesapeaki* single-infections are restricted in Gujan and IAO stations with very low prevalence, 14 % and 2 % respectively, combined with low infection intensities. It is common that one pathogen influences the acquisition of and/or the infection dynamics by a second opportunistic pathogen (Susi *et al.*, 2015). Two hypotheses could be formulated about the occurrence of these two parasites in clam tissue samples: **1)** *P. olseni* may play a facilitating role in the *P. chesapeaki* infection of Manila clam. Settlement of a first parasite can benefit to a second parasite because host is already immune-compromised by the first infection; **2)** *P. chesapeaki* infection can be completely independent to *P. olseni* infection. The occurrence of *P. chesapeaki* single-infection could indicate a non-obligatory relationship. These two hypotheses are supported because *R.*

philippinarum may be a "sub-optimal host" and/or reservoir host for *P. chesapeakei* whose occurrence would be the result of a secondary opportunistic infection. In the Chesapeake Bay, *P. marinus* and *P. chesapeakei* infect two sympatric host bivalves and are sporadically detected in co-infection within the same host although in low prevalence and distribution in oysters for *P. chesapeakei* and conversely in clams for *P. marinus* (Reece *et al.*, 2008). Dungan *et al.*, (2002) suggested that *P. marinus* can infect *Mya arenaria* as an alternative host from which it can be transmitted back to oysters. Hence, in Chesapeake Bay, the clam *Mya arenaria* is suspected to be the primary host of *P. chesapeakei* with high prevalences and infection intensities (McLaughlin *et al.*, 2000a; Reece *et al.*, 2008). Ramilo *et al.*, (2016) described a new association of *P. chesapeakei* with *P. olseni* as a mismatch or a punctual introduction or a bad adaptation to a "new" host supporting above hypotheses.

4.2. Co-infection: a patchy distribution at individual scale

Among co-infected clams, *P. olseni* infection appears homogenous across all tissue samples compared to *P. chesapeakei* which is often restricted to one or two organs. When *P. chesapeakei* tended to inhabit the same host as *P. olseni*, it is segregated in one or two organs of the Manila clams. We identified here three major infection patterns of co-infected clams showing moderate to low infection intensities: **1)** *P. chesapeakei* infection is mainly prevalent in gill in the Piquey station (n=11/18 clams) and ranged from 5.0 to 6.4 log of copy number per gram of wet tissue in; **2)** adductor muscle mainly in Gujan (n=11/11) and IAO (n=8/15); **3)** *P. chesapeakei* displayed a moderate level of infection in digestive gland, mantle and remaining tissue (5.9 to 6.9 log of copy number.g⁻¹ of wet tissue) distributed homogeneously across stations. The high variability of co-infection groups, in terms of organ(s) infected or stations, highlights that *P. chesapeakei* may be more affected by local biotic or abiotic factors than *P. olseni*. Hence co-infection patterns could be the result of a complex overlap between individual infection level and environmental factors (e.g. sediment type, localisation in the basin, temperature, salinity). Finally, 98 % of co-infected hosts are associated with moderate *P. olseni* infection intensities. A positive relationship links *P. olseni* and *P. chesapeakei* infection intensities when they live in sympatry in the same organ. However, this relationship theoretically demonstrates that we don't detect *P. chesapeakei* when infection intensity of *P. olseni* is under 2.2 log copy number. g⁻¹ of wet tissue. Because of its burden, *P. olseni* is largely dominant in the co-infected organs. This recurrent observation of *P. olseni* dominance in co-infected organs of *R. philippinarum* is in

accordance with apparent results obtained by Arzul *et al.* in 2014 on the *Ruditapes decussatus* in the Leucate lagoon (Mediterranean Sea). However, in the same study, the opposite pattern with a dominance of *P. chesapeaki* was observed at Bonne Anse in *R. philippinarum*. We could therefore hypothesise that *P. chesapeaki* infections may be facilitated by moderate infection of *P. olseni* which could increase transepithelial migration of hemocytes as described for *P. marinus* in *C. virginica* (Lau *et al.*, 2018) or could reduce the efficiency of defensive mechanisms of the clams similarly to secondary infections by bacteria or virus (Montes *et al.*, 2001). Hence, our results suggest a competitive exclusion of *P. chesapeaki* parasites in case of high infection intensity of *P. olseni* because of a direct or indirect interspecific competition for resource or space.

Interestingly, in all sampling stations, *P. chesapeaki* was never detected in the foot. This observation is not a PCR amplification bias because *P. chesapeaki* was detected in artificial infection with a mix of plasmid containing *P. chesapeaki* ITS sequence and host foot gDNA (see Itoiz *et al.* submitted). The foot is one of the last organ infected by *P. olseni* when the infection intensity is heavy (Wang *et al.*, 2018). Hence, if *P. chesapeaki* is a secondary invader as previously hypothesis, the detection of *P. chesapeaki* cells may correspond to an early infection or *P. chesapeaki* is not able to infect the foot compartment as *P. olseni* does. Sporadic detections of *P. chesapeaki* were also confirmed by histology techniques in different *in situ* studies on co-infection (Reece *et al.*, 2008; Arzul *et al.*, 2012; Ramilo *et al.*, 2016). *P. chesapeaki* was described in digestive gland of two *R. philippinarum* by Ramilo *et al.* (2016) while detections in digestive gland and gonadal tissue were also observed in *R. decussatus* (Arzul *et al.*, 2012). Conversely, in other bivalves, as *Mya arenaria* or *Cyrtopleura costata*, infection in gills was also detected but very localised and concentrated per compact cell clusters (Reece *et al.*, 2008). Low spreading and punctual spot of infection in *Ruditapes* species maintains the hypothesis of “sub-optimal” host for *P. chesapeaki*.

The variability in organ infected by *P. chesapeaki* could confirm that local conditions can influence at small scale co-infection process. Surprisingly, the Gujan station showed an unexpected *P. chesapeaki* infection pattern with only one organ infected, the adductor muscle. Dang *et al.* (2008) and Binias *et al.* (2014a) hypothesise, in case of the BMD, that posterior muscle located nearest the sediment surface was more vulnerable to certain pathogenic agents or environmental variations. Sediment could be a potential reservoir of *Perkinsus*

contamination for clams (Choi, 2002). Thus, muddy sediments could retain *Perkinsus* hypnospores (Binias *et al.*, 2014a) and favour contamination by this stage of life.

Previous publication highlighted that environmental conditions play a key role on developmental stages, including transformation from trophozoites to pre-zoosporangia, and the survival of zoospores should be evaluated to better understand the co-infection repartition in hosts (Umeda and Yoshinaga, 2012). Hence, gills co-infection in Piquey could be explained by two hypotheses: **1)** the proximity to mouth of the bay. Due to its localisation, Piquey is more influenced by oceanic current than the inner part of the bay (input of freshwater) (Dang *et al.*, 2010b). These oceanic conditions may favour *P. chesapeaki* zoosporulation, which has an optimal of salinity higher than *P. olseni* (La Peyre *et al.*, 2006). In addition a size-effect on Perkinsosis infection were noticed by Dang *et al.* (2010b), explaining that clams exceeding 20 mm shell length are more heavily infected. Piquey harbours the highest shell length which may favour parasites load due to a higher clearance rate; and **2)** Piquey area harbour preferential hosts (not determined) for *P. chesapeaki* which contribute to its dissemination.

5. Conclusion

The recent detection of two *Perkinsus* species highlights a key element in dynamic of cryptic infections in host clam populations. In this study, global *Perkinsus* infections are led by *P. olseni* and exposed an environmental gradient corresponding to its optimal proliferation. Conversely, *P. chesapeaki* is very localised and occasional in Manila clams suggesting a more secondary role in these infections. Low prevalences of co-infection and *P. chesapeaki* single-infection support that *R. philippinarum* may not be its preferred host. To date, it is not possible to conclude whether *P. olseni* facilitates the opportunistic infection of *P. chesapeaki* or whether this secondary infection is independent. Taken all together, these data support the hypothesis of introduction event of *P. chesapeaki* in Europe with other bivalves' species, *Mya arenaria* or *Mercenaria mercenaria*, and confirms the urgent need to monitor these parasites which are able to spillover in new indigenous host populations. It is now urgent and timely to expand our view of the host range of *Perkinsus* species and investigate the sympatric benthic fauna for hot-spots of co-infections. In the light of these results, co-infection process is a Pandora box that justifies the need to shift from the study of isolated pathogens to a more integrated approach.

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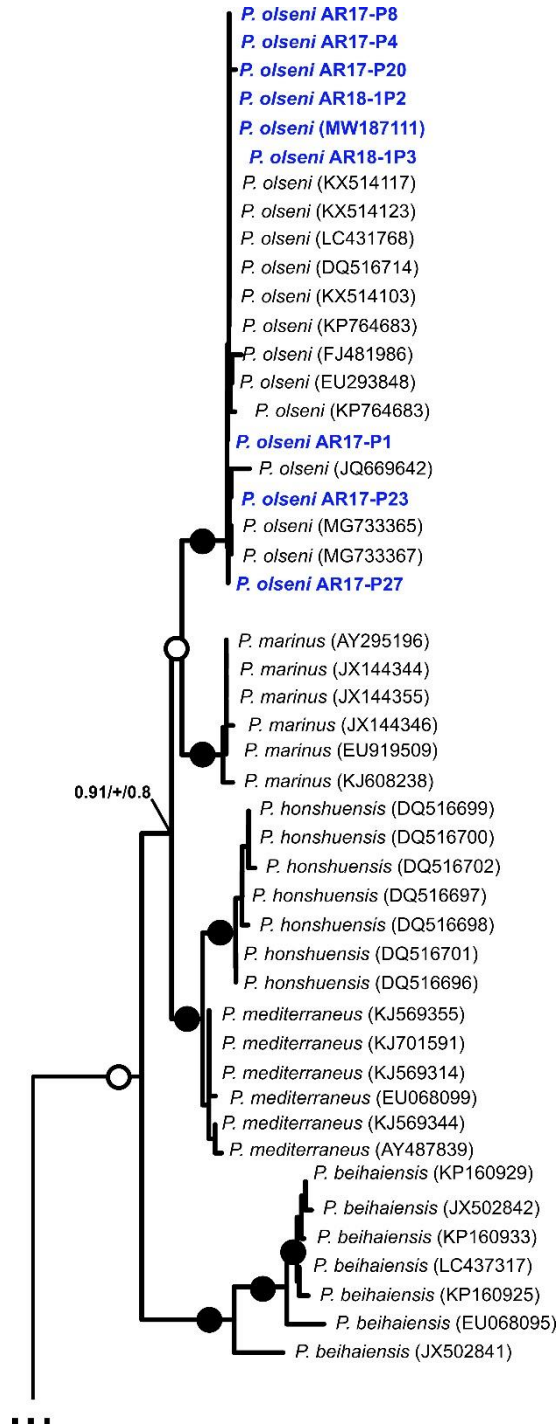
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Chapitre 2.2 : Supplementary



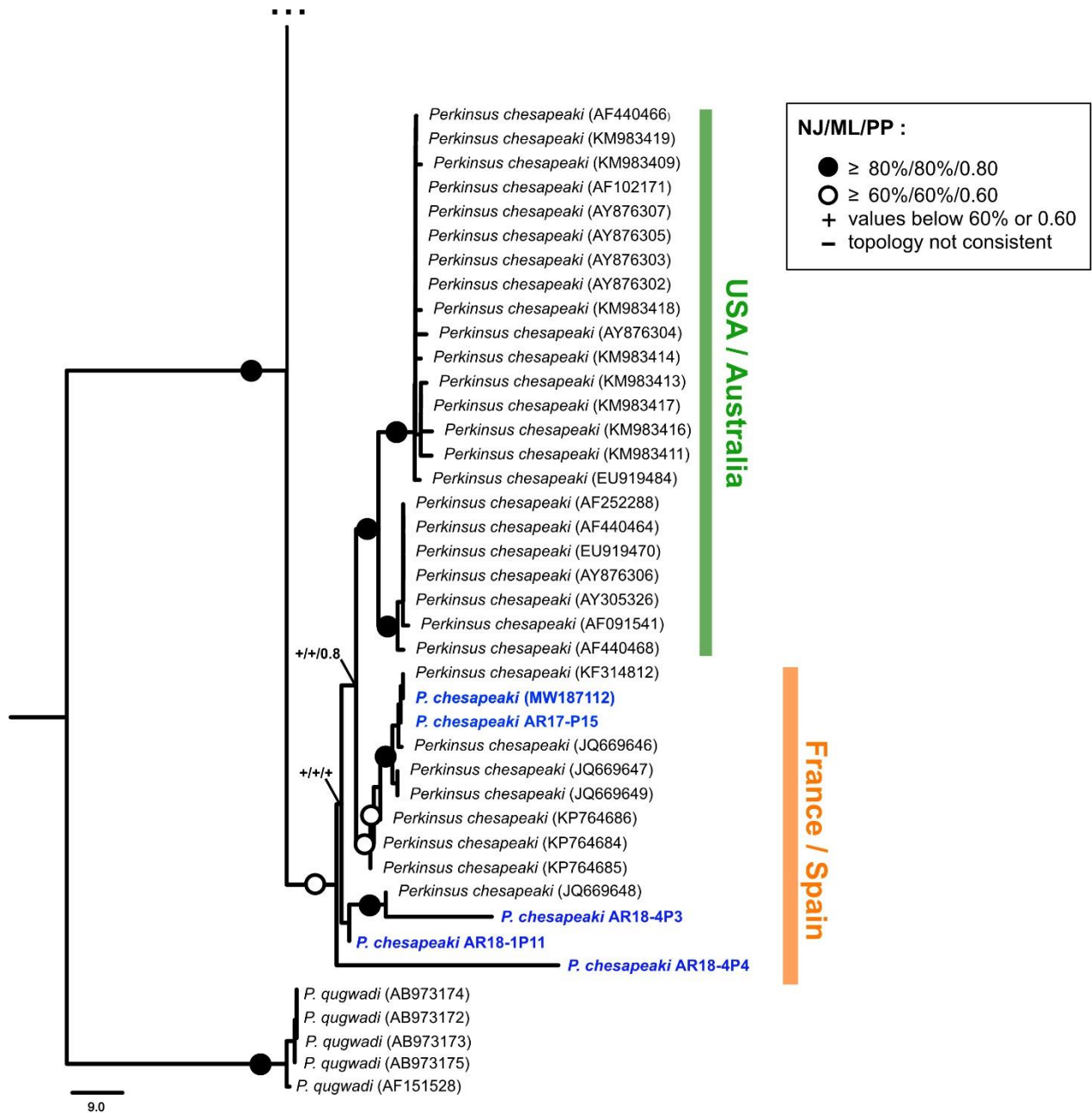


Figure S1. *Perkinsus* ITS1-5.8S-ITS2 phylogeny including *Perkinsus* sequences from Arcachon bay. Neighbour joining (NJ) topology was represented and composed of 55 sequences of seven *Perkinsus* species from Genbank and 14 sequences from Arcachon bay (sampling 2017 and 2018). The phylogeny was calculated from 69 taxa and 472 characters alignment position. NJ bootstrap values (1 000 replicates), ML bootstrap values (1 000 replicates) and Bayesian posterior probability (2 000 000 generations, GTR+ Γ model) were notated using the following convention: support values are summarized by \bullet when $\geq 80\%/0.8$ and white circle when it is not the case but values $\geq 60\%/0.6$. When support value is $< 60\%/0.6$ but topology is consistent, the support value is summarized by a '+' and conversely by a '-' if the topology is not consistent. Five sequences of *P. qugwadi* were used to root the tree. Sequences from *Perkinsus* culture were represented in blue.

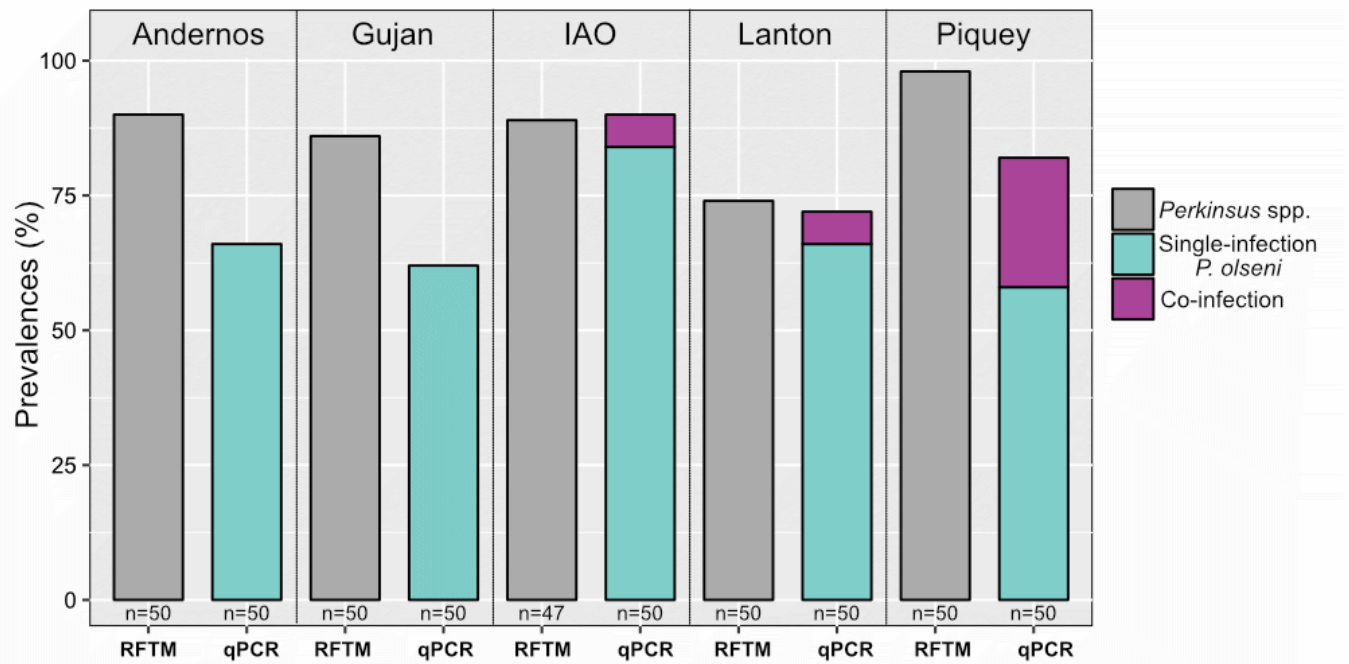


Figure S2. Prevalences of *Perkinsus* species evaluated by RFTM and duplex qPCR assay on gill tissues. Each prevalence using both methodologies was determined on a total of 50 clams except for Ile aux Oiseaux (IAO) where the RFTM counting was based on 47 clams.

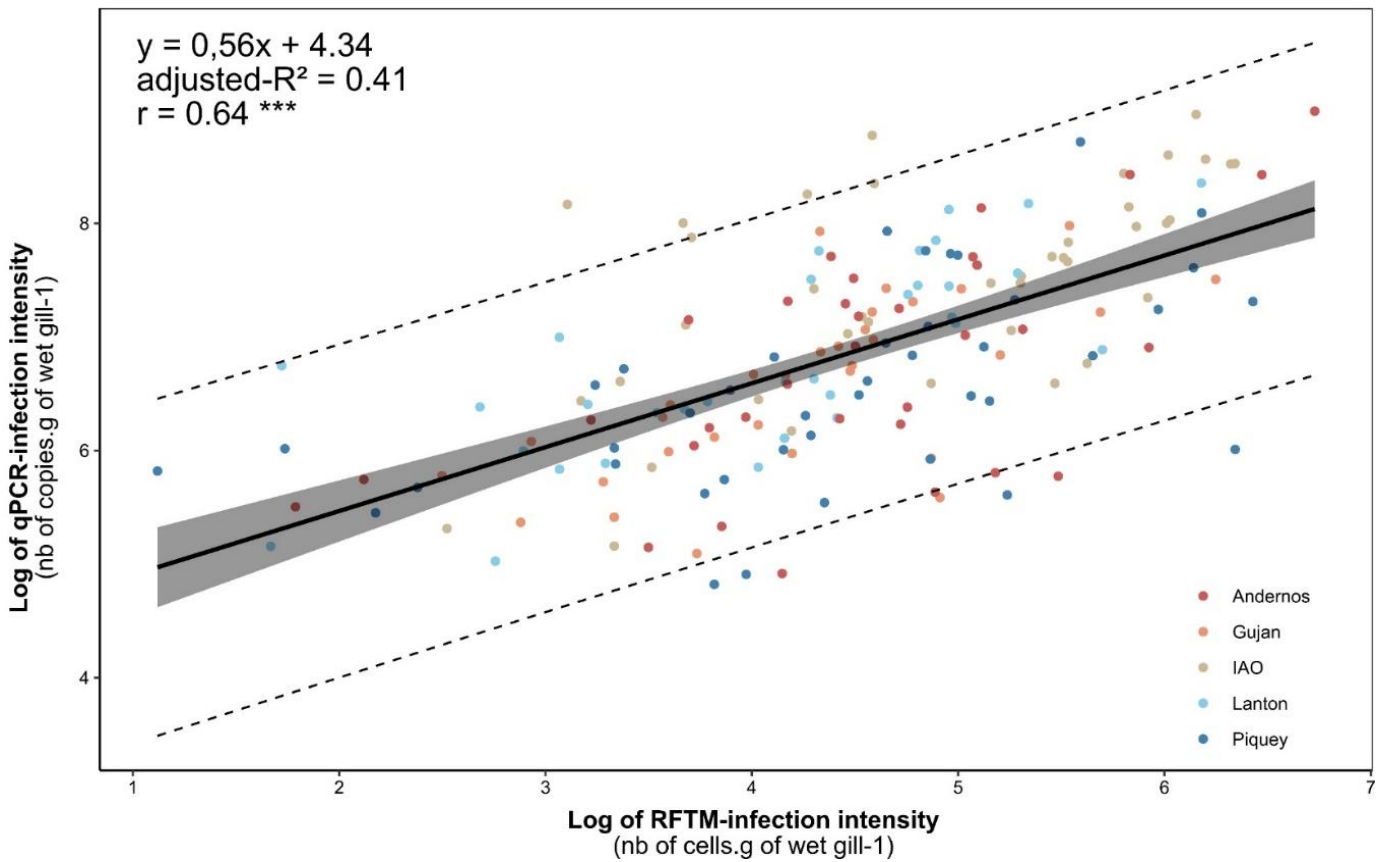


Figure S3. Linear regressions among Ct values, infection intensities determined by RFTM assay (nb. of cells.g⁻¹ of wet gill) and by qPCR assay (nb. of copies.g⁻¹ of wet gill). The infection intensities determined by RFTM are based on the global counting of *Perkinsus* sp. hyphospores in gills (n= 250). The infection intensities determined by duplex qPCR are based on the sum of *P. olseni* and *P. chesapeaki* copies in gills. RFTM and qPCR relationship: $y=0.56x+4.34$. Ct: Cycle threshold value. r: Pearson's coefficient.

Table S1. 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	<i>Perkinsus</i>	<i>marinus</i>	60_2	(da Silva <i>et al.</i> , 2013)
KJ608238	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>marinus</i>	70_3_C_c5	(da Silva <i>et al.</i> , 2014)
JX144355	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>marinus</i>	69	(da Silva <i>et al.</i> , 2013)
EU919509	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>marinus</i>	PXBICv25-B9-C5	(Reece <i>et al.</i> , 2008)
AY295196	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>marinus</i>	SC2_4_7	(Brown <i>et al.</i> , 2004)
JX144344	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>marinus</i>	60	(da Silva <i>et al.</i> , 2013)
KJ569344	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>mediterraneus</i>	H08_3_06	(Valencia <i>et al.</i> , 2014)
AY487839	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>mediterraneus</i>	D10	(Casas <i>et al.</i> , 2004)
KJ569314	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>mediterraneus</i>	H03_3_04	(Valencia <i>et al.</i> , 2014)
KJ569355	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>mediterraneus</i>	H03_28_A3	(Valencia <i>et al.</i> , 2014)
EU068099	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>mediterraneus</i>	Jer2_50	(Casas <i>et al.</i> , 2008)
KJ701591	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>mediterraneus</i>	12	(Ramilo <i>et al.</i> , 2015) (Dungan and Reece, 2006)
DQ516701	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>honshuensis</i>	Mie3gH8_3	(Dungan and Reece, 2006)
DQ516696	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>honshuensis</i>	Mie3g_2	(Dungan and Reece, 2006)
DQ516702	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>honshuensis</i>	Mie3gH8_4	(Dungan and Reece, 2006)
DQ516699	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>honshuensis</i>	Mie3gH8_1	(Dungan and Reece, 2006)
DQ516700	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>honshuensis</i>	Mie3gH8_2	(Dungan and Reece, 2006)
DQ516698	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>honshuensis</i>	Mie3g_4	(Dungan and Reece, 2006)
DQ516697	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>honshuensis</i>	Mie3g_3	(Dungan and Reece, 2006)
JX502841	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>beihaiensis</i>	138	(Sabry <i>et al.</i> , 2013)
KP160933	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>beihaiensis</i>	CBMA287	(Queiroga <i>et al.</i> , 2015) Itoh <i>et al.</i> 2019
LC437317	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>beihaiensis</i>	Koitogawa_4	unpublished
KP160925	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>beihaiensis</i>	CBMA92	(Queiroga <i>et al.</i> , 2015)
KP160929	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>beihaiensis</i>	CBMA142	(Queiroga <i>et al.</i> , 2015)
JX502842	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>beihaiensis</i>	34	(Sabry <i>et al.</i> , 2013)
EU068095	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>beihaiensis</i>	QZ0649	(Moss <i>et al.</i> , 2008)
AB973173	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>qugwadi</i>	8302-1	(Itoh <i>et al.</i> , 2013)
AB973174	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>qugwadi</i>	8302-11	(Itoh <i>et al.</i> , 2013)
AB973175	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>qugwadi</i>	8302-51	(Itoh <i>et al.</i> , 2013)
AB973172	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>qugwadi</i>	6675-2	(Itoh <i>et al.</i> , 2013) Hervio <i>et al.</i> 1999
AF151528	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>qugwadi</i>	-	unpublished
AF440466	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>chesapeaki</i>	ATCC 50864	(Dungan <i>et al.</i> , 2002)

Chapitre 2.2 : Occurrence et distribution de la co-infection entre *P. olseni* et *P. chesapeaki*

KM983419	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>chesapeaki</i>	QLD1_8cl	Dang <i>et al.</i> 2014 (unpublished)
KM983409	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>chesapeaki</i>	QLD2_C3	Dang <i>et al.</i> 2014 (unpublished)
KM983418	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>chesapeaki</i>	QLD1_A4	Dang <i>et al.</i> 2014 (unpublished)
KM983413	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>chesapeaki</i>	QLD1_B8	Dang <i>et al.</i> 2014 (unpublished)
KM983414	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>chesapeaki</i>	QLD1_B7	Dang <i>et al.</i> 2014 (unpublished)
KM983417	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>chesapeaki</i>	QLD1_A6	Dang <i>et al.</i> 2014 (unpublished)
KM983416	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>chesapeaki</i>	QLD1_B3	Dang <i>et al.</i> 2014 (unpublished)
KM983411	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>chesapeaki</i>	QLD2_C1	Dang <i>et al.</i> 2014 (unpublished)
AF102171	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>chesapeaki</i>	-	(Coss <i>et al.</i> , 2001)
AY876307	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>chesapeaki</i>	-	(Coss <i>et al.</i> , 2001)
AY876305	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>chesapeaki</i>	ATCC_50807 cl5	(Burreson <i>et al.</i> , 2005)
AY876303	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>chesapeaki</i>	ATCC_50807 cl2	(Burreson <i>et al.</i> , 2005)
AY876302	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>chesapeaki</i>	ATCC_50807 cl1	(Burreson <i>et al.</i> , 2005)
AY876304	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>chesapeaki</i>	ATCC_50807 cl3	(Burreson <i>et al.</i> , 2005)
AY876306	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>chesapeaki</i>	ATCC_50807 cl6	(Burreson <i>et al.</i> , 2005)
EU919484	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>chesapeaki</i>	PXBIMa5-G1-D12	Reece <i>et al.</i> 2008 (unpublished)
EU919470	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>chesapeaki</i>	EBPIMa2-C10-E1	Reece <i>et al.</i> 2008 (unpublished)
AF252288	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>chesapeaki</i>	CCA2001	(Coss <i>et al.</i> , 2001)
AF440464	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>chesapeaki</i>	CRMA-J44/E3	(Dungan <i>et al.</i> , 2002)
AF440468	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>chesapeaki</i>	CRTP-17/A10	(Dungan <i>et al.</i> , 2002)
AY305326	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>chesapeaki</i>	ATCC 50807	(Pecher <i>et al.</i> , 2004)
AF091541	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>chesapeaki</i>	G117	(Kotob <i>et al.</i> , 1999) Carrasco <i>et al.</i> 2013
KF314812	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>chesapeaki</i>	-	(unpublished)
JQ669646	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>chesapeaki</i>	10154_31_3/6_1	(Arzul <i>et al.</i> , 2012)
JQ669647	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>chesapeaki</i>	10154_31_2	(Arzul <i>et al.</i> , 2012)
JQ669649	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>chesapeaki</i>	10154_31_1	(Arzul <i>et al.</i> , 2012)
JQ669648	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>chesapeaki</i>	08143_1_1	(Arzul <i>et al.</i> , 2012)
KP764686	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>chesapeaki</i>	Isolate 3	(Ramilo <i>et al.</i> , 2015)
KP764685	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>chesapeaki</i>	Isolate 2	(Ramilo <i>et al.</i> , 2015)
KP764684	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>chesapeaki</i>	Isolate 1	(Ramilo <i>et al.</i> , 2015)
MW187112	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>chesapeaki</i>	AR18_1G3F	Itoiz <i>et al.</i> (submitted)
JQ669642	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>olseni</i>	05067_3P2/1_6	(Arzul <i>et al.</i> , 2012)
KX514117	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>olseni</i>	MS2-2	Cho <i>et al.</i> 2018 unpublished
KX514123	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>olseni</i>	SS2-2	Cho <i>et al.</i> 2018 unpublished

Chapitre 2.2 : Occurrence et distribution de la co-infection entre *P. olsenii* et *P. chesapeaki*

LC431768	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>olsenii</i>	shoG	Imajoh <i>et al.</i> 2018 unpublished (Dungan and Reece, 2006)
DQ516714	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>olsenii</i>	Mie13v_8	(Shamal <i>et al.</i> , 2018)
MG733367	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>olsenii</i>	PM51DH1	(Shamal <i>et al.</i> , 2018)
MG733365	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>olsenii</i>	PM45EL4	(Shamal <i>et al.</i> , 2018)
KP764683	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>olsenii</i>	8	(Ramilo <i>et al.</i> , 2016) Cho <i>et al.</i> 2018 unpublished
KX514103	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>olsenii</i>	WD4-2	(Ramilo <i>et al.</i> , 2016)
KP764681	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>olsenii</i>	6	Elandaloussi <i>et al.</i> , 2008 unpublished
FJ481986	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>olsenii</i>	Rp1	(Elandaloussi <i>et al.</i> , 2009)
EU293848	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>olsenii</i>	-	Itoiz <i>et al.</i> (submitted)
MW187111	Perkinsea	Perkinsida	<i>Perkinsus</i>	<i>olsenii</i>	AR18_F1F10	

Table S2. Concordance and discordance between RFTM and qPCR methodology applied on gill tissue samples.

Station	Discordance (%)	Concordance (%)
IAO	14	86
Andernos	24	76
Gujan	24	76
Lanton	14	86
Piquey	16	84

Table S3. Mean infection intensities of *P. olseni* and *P. chesapeaki* in common co-infected organs. Means and standard-deviation (sd) are in number of copies per gram of wet tissue samples (Mean \pm sd). n: the number of common organ infected by *P. olseni* and *P. chesapeaki*. Add. muscle: Adductor muscle; Rem. tissue: Remaining tissue.

		Mean infection intensities (nb. of copies.g ⁻¹ of wet tissue)		
		n	<i>P. olseni</i>	<i>P. chesapeaki</i>
IAO	Gill	3	2,02x10 ⁶ \pm 1,63x10 ⁶	6,02x10 ⁵ \pm 6,89x10 ⁵
	Digestive gland	2	6,90x10 ⁷	2,94x10 ⁶
	Add. muscle	8	1,28x10 ⁷ \pm 1,36x10 ⁷	7,45x10 ⁵ \pm 5,75x10 ⁵
	Mantle	1	4,60x10 ⁷	2,33x10 ⁶
	Rem. tissue	6	5,60x10 ⁷ \pm 4,76x10 ⁷	3,33x10 ⁶ \pm 2,79x10 ⁶
Gujan	Add. muscle	9	3,69x10 ⁵ \pm 5,27x10 ⁵	7,10x10 ⁴ \pm 5,81x10 ⁴
Piquey	Gill	12	1,41x10 ⁷ \pm 2,62x10 ⁷	6,12x10 ⁵ \pm 7,64x10 ⁵
	Digestive gland	3	1,05x10 ⁷ \pm 7,60x10 ⁶	7,67x10 ⁵ \pm 7,94x10 ⁵
	Add. muscle	2	3,61x10 ⁶	3,27x10 ⁵
	Mantle	2	4,69x10 ⁷	3,03x10 ⁶
	Rem. tissue	1	1,29x10 ⁷	8,76x10 ⁵
Lanton	Gill	3	5,25x10 ⁶ \pm 6,51x10 ⁶	2,32x10 ⁵ \pm 1,56x10 ⁵
	Digestive gland	2	1,11x10 ⁸	4,58x10 ⁵
	Add. muscle	1	2,17x10 ⁶	3,87x10 ⁵
	Rem. tissue	1	6,10x10 ⁷	2,11x10 ⁶

Chapitre 2.2 : Supplementary - References

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Chapitre 2.3 : Génomes de deux parasites, *P. olseni* et *P. chesapeakei* (Perkinsozoa), co-infectant la palourde japonaise, *Ruditapes philippinarum*, dans le bassin d'Arcachon.

Avant-propos

Au cours du **Chapitre 2.2**, nous avons vu que les infections globales à *Perkinsus* étaient majoritairement influencées par un gradient environnemental correspondant à l'optimal d'infection de *P. olseni*. Alors que *P. olseni* domine largement ces cas d'infection, *P. chesapeakei* reste très localisé chez les palourdes japonaises, ce qui suggère un rôle assez secondaire de cette infection. Les faibles prévalences de la co-infection et de l'infection unique par *P. chesapeakei* confirment que *R. philippinarum* n'est peut-être pas un hôte préférentiel pour son développement. Cependant, il n'est pas possible de conclure définitivement sur le rôle de chacun des deux parasites dans ces co-infections, même si les résultats globaux penchent majoritairement en faveur d'un rôle de facilitation par *P. olseni* qui permet l'infection opportuniste de *P. chesapeakei*. Prises dans leur ensemble, ces données soutiennent l'hypothèse d'une possible introduction de *P. chesapeakei* en Europe et confirment le besoin urgent de surveiller ces parasites qui sont capables de se répandre dans de nouvelles populations hôtes indigènes. Ainsi, nous nous intéressons à décrypter qu'elles sont les caractéristiques métaboliques et de virulences de chacune des deux espèces. Dans ce **Chapitre 2.3**, nous avons séquencé les génomes et transcriptomes de *P. olseni* et *P. chesapeakei* à partir de cultures monoclonales isolées de palourdes japonaises dans le bassin d'Arcachon. Nous présentons, ici, les premières ébauches de séquences génomiques de ces deux espèces de *Perkinsus* proliférant en Europe. Les génomes ont été séquencés en utilisant en short-reads (Illumina HiSeq) et en long-reads (Oxford Nanopore) afin d'assurer une bonne qualité des assemblages génomiques. Les travaux sur ces génomes sont toujours en cours d'analyse.

Draft genomes sequence of the two Perkinsozoa parasites, *P. olseni* and *P. chesapeaki*, co-infecting *Ruditapes philippinarum* isolated from Arcachon Bay (France)

Abstract

Perkinsosis disease, caused by the aetiological agent *Perkinsus olseni* (Perkinsozoa, Alveolata), is a major threat for shellfish because it is responsible for mortality event in clam populations mostly Asia and in South Atlantic coastline (Portugal and Spain). In Europe, especially in Arcachon Bay (France), this parasite was detected in association with another *Perkinsus* species, *P. chesapeaki*, which was never associated with mortality event. Here, we report the first draft genomic sequences of these two European *Perkinsus* species, which co-infect the Manila clam, *Ruditapes philippinarum* in the Arcachon Bay (France). Genomes were sequenced using both short-read (Illumina HiSeq) and long-read (Oxford Nanopore) platforms to improve the genomic assemblies. The genome assembly of *P. olseni* was composed of 48 contigs (>100kb) with a cumulated length of 50 Mb. The genome assembly of *P. chesapeaki* was composed of 46 contigs (>100 kb) with a cumulated length of 49 Mb.

Perkinsus olseni and *Perkinsus chesapeaki* belong to the Perkinsidae (Perkinsozoa, Alveolata) which includes broad host range parasites of molluscs, dinoflagellates and amphibians. The parasite *P. olseni* infects mostly mollusc species and has been on the O.I.E-list of diseases since 2006 because it is considered as an emerging pathogen threatening ecosystems and aquaculture sustainability. In Europe, it occurred regularly along the Atlantic coastline, causing mortalities in Portugal, Italy and Spain (Azevedo, 1989; Figueras *et al.*, 1992; Pretto *et al.*, 2014). Another species, *P. chesapeaki*, which was never associated with mortality event, was recently detected along the European Atlantic coastline and in association with the exotic species *P. olseni* (Arzul *et al.*, 2012)(Itoiz *et al.*, in prep). It is now presumed that *P. olseni* and *P. chesapeaki* have been co-introduced with their host for aquaculture purpose from Asia and North-America respectively (Arzul *et al.*, 2012; Ruano *et al.*, 2015).

In 2009, the first *Perkinsus marinus* genome was sequenced by Sanger sequencing (GCA_000006405). In 2020, using both Illumina and Nanopore technology, new genomes of Australian strains of *P. olseni* and *P. chesapeaki* were sequenced (Bogema *et al.*, 2020). However, the assembly quality of these draft genomes is still poorly resolved for *P. olseni* (Size: from 39.9 to 90.45 Mb, scaffold: from 1,358 to 41,490 Mb) and *P. chesapeaki* (Size: 41 Mb, scaffold 3,544 Mb, N50: 34,192 Mb) and might not reflect the diversity of the parasitic strains spreading along the European Atlantic coastline. The intensification of the sequencing effort testifies of the growing interest for the *Perkinsus* parasites worldwide, which is now urgent to be addressed in a context of the sustainable conservation policies for managing global diversity. Here we present draft genomes of European strains of *P. olseni* and *P. chesapeaki* infecting the Manila clams and isolated from the same sampling site in Arcachon Bay (France).

Perkinsus spp. cultures were established from infected Manila clams, *R. philippinarum*, sampled in the Arcachon bay on the 7th and 8th of November 2018 (South-West of France, Atlantic coast, 44°41'60" N;1°10' W). Cultures were initiated following a simplified procedure according to Casas *et al.* (2002) where gills were excised and decontaminated with several successive baths of antibiotic solution (containing (l⁻¹) 400 000 U ampicillin, 0.4 g streptomycin sulphate, 0.4 g kanamycin, 0.2 g gentamycin) and sterile seawater. Gill tissues were incubated in *Perkinsus* medium broth (ATCC medium 1886) at 25 °C for 10-

15 days up to the release of trophozoites. Monoclonal strains were obtained from *P. olseni* and *P. chesapeakei* cultures isolated from Lanton. Following the agar-based method developed by Cold *et al.* (2016), *P. olseni* and *P. chesapeakei* cultures were diluted in Perkinsus medium broth (ATCC medium 1886) at 2,000 cell.ml⁻¹ before plating onto solid medium made of Perkinsus medium broth (ATCC medium 1886) implemented with 0.75 % agar. Agar plates were incubated at 25 °C until first visible colonies. Finally, each colony was checked using an inverted light microscope and subcloned twice using the same methodology before seeding into liquid Perkinsus broth medium (ATCC medium 1886). Once cultures well established, a PCR assay was carried out using species-specific primers targeting the Internal Transcribed Spacer 2 (ITS2) specifically of *P. olseni* and *P. chesapeakei* respectively: P_{olsITS2_F}/P_{olsITS2_R} (*P. olseni*) and P_{chesITS2_F}/P_{chesITS2_R} (*P. chesapeakei*) (Itoiz *et al.*, submitted). Amplicons were visualised by electrophoresis and sequenced for species confirmation using Eurofins sequencing company.

Large volume of trophozoite cultures of *P. olseni* clone S11G3F and *P. chesapeakei* clone S11F10F were prepared and harvested in the log-phase to perform DNA extraction. The genomic DNA of *P. olseni* (S11G3F) and *P. chesapeakei* (S11F10F) was extracted using CTAB-based DNA extraction method adapted from Winnepenninckx *et al.* (1993) as follows. Approximately, 2.5 x 10⁶ cells of *P. olseni* (F1F10) and 3.2 x 10⁶ cells of *P. chesapeakei* (1G3F) (determined using the gold standard method based on Ray's Fluid Thioglycollate Medium recommended by the Organisation for Animal Health) were pelleted for 15 minutes at 4 000 rpm and rinsed twice in sterile PBS 1 X. Pelleted cells were frozen 5 times consecutively in liquid nitrogen and incubated for 1 h at 37 °C in CTAB buffer (2 % CTAB, 100 mM TrisHCl pH=8.0, 20 mM EDTA, 1.4 mM NaCl) containing 0.1 mg.ml⁻¹ proteinase K, 5mg.ml⁻¹ lysozyme and 1 % of sodium lauroyl sarcosinate. Cell mixtures were slightly ground with a pestle in 1.5 ml microcentrifuge tube and incubated for 45 minutes at 60 °C adding 0.2 % of β-mercaptoethanol. Lysis mixtures were mixed with an equal volume of chloroform/isoamylalcohol (24:1) and centrifuged. The aqueous phase was treated with RNase solution for 30 minutes at 37 °C prior to DNA precipitation with cold isopropanol. DNA were pelleted and rinsed with cold 76 % ethanol/10 mM ammonium acetate to finally be dried at room temperature and resuspended in 10 mM Tris buffer. Genomic DNA were quantified on the Qubit fluorometer with the Qubit HS

dsDNA Assay kit (Invitrogen™). The quality was assessed by running DNA on a 0.8 % agarose gel electrophoresis and measuring absorbance ratio A260/A280 with the spectrophotometer NanoDrop 8 000 (Thermo Scientific).

The nanopore library for genomic DNA was prepared according to Native barcoding genomic DNA protocol from the Oxford Nanopore Technologies (ONT). Briefly, the whole procedure is as follow: the end repair process is completed by mixing 1 to 2 µg of genomic DNA with NEBNext End repair/dA-tailing Module (New England Biolabs) and NEBNext FFPE DNA Repair kit (New England Biolabs). Mix was incubated in thermal cycler at 20 °C for 20 minutes and 65 °C for 5 minutes. The end-prep reaction DNA were purified by addition of 60 µl of Agencourt AMPure XP beads (Beckman Coulter) and eluted in 25 µl of nuclease-free water. Recovery was evaluated using Qubit fluorometer and each end-prep sample was diluted to 500 ng in 22.5 µl of nuclease-free water. *P. olseni* and *P. chesapeaki* samples were specifically barcoded using the Native Barcoding Expansion kit (EXP-NBD104; ONT). Reaction prep were incubated 10 minutes at room temperature and purified by adding 50 µl of Agencourt AMPure XP beads. Barcoded DNA were quantified using Qubit fluorometer and then pooled in equimolar amounts (350 ng per sample barcoded) to reach 700 ng in 65 µl of nuclease-free water. Pooled barcoded DNA were ligated with the NEBNext Quick Ligation Module (New England Biolabs) and Ligation Sequencing Kit (ONT; SQK-LSK109). For the last purification, the reaction mix were incubated 10 minutes at room temperature using Agencourt AMPure XP beads. The beads were washed twice with 250 µl of Long Fragment Buffer to enrich for DNA fragments of 3 kb or longer. Pellets were resuspended in elution buffer (ONT; providing in kit SQK-LSK109) and incubated 10 minutes at 37 °C to improve the recovery of long fragments. Finally, the eluate was recovered to provide the final sequencing library. Before loading, the library and the flow cell were prepared using the flow cell priming kit (ONT; EXP-FLP002) according to ONT protocol. The flow cell (Rev-D, 1 097 pores available) was inserted in a MinION device and equilibrated with priming mix for 30 minutes. The prepared library was loaded into the flow cell. The run was performed for 18h. The Illumina HiSeq 2 x 250 bp sequencing were processed for both genomes by GENEWIZ (Germany GmbH, Leipzig) and yielded 12,665,673 reads for *P. olseni* and 11,840,131 reads for *P. chesapeaki*.

The Illumina sequencing of genomes was adapter and quality trimmed with Trimmomatic v. 0.32 (Bolger *et al.*, 2014). A total of 12,004,666 reads for *P. olseni* and 10,582,889 reads for *P. chesapeaki* was retained. Long-reads from MinION sequencing were quality and length filtered with NanoFilt v2.6 (length 500 bp; quality 7). Then, genomes were *de novo* assembled using Canu v. 2.0 (Koren *et al.*, 2017) and two rounds of Pilon v. 1.23 for the polishing using the previous trimmed Illumina reads (Walker *et al.*, 2014).

- ***P. olseni* assembly:** The final assembly had a total length of 50,465,104 bp, a coverage of 29 and an N₅₀ of 1,526,217 bp (L₅₀, 9). Total of 48 contigs are larger than 100 kb with a maximum length of 5,059,513 bp. The GC content is 51.6 %, a percentage similar to that of *P. olseni* (assembly: ASM1311513v1) (51.5 %) but higher than that of *P. chesapeaki* (assembly: ASM1311514v1) (46.5 %) and *P. marinus* (assembly: GCA_000006405) (47.4 %). Genome completeness was assessed using BUSCO v.2 (Simão *et al.*, 2015). Of the 171 alveolate (lineage alveolata_odb10, 65 species) core genes, 91 % were completely (83 % single-copy, 4 % duplicated) or partially (4 %) recovered in the assembly.

- ***P. chesapeaki* assembly:** The final assembly had a total length of 49,035,408 bp, a coverage of 25, and an N₅₀ of 2,188,628 bp (L₅₀, 7). Total of 46 contigs are larger than 100 kb with a maximum length of 5,392,088 bp. The GC content is 44.1 %, a percentage similar to that of *P. chesapeaki* (assembly: ASM1311514v1) (46.5 %) but lower than that of *P. marinus* (assembly: GCA_000006405) (47.4 %) and *P. olseni* (assembly: ASM1311513v1) (51.5 %). Genome completeness was assessed using BUSCO v.2 (Simão *et al.*, 2015). Of the 171 alveolate (lineage alveolata_odb10, 65 species) core genes, 81 % were completely (68 % single-copy, 3 % duplicated) or partially (10 %) recovered in the assembly.

Chapitre 2.3 : References

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DISCUSSION GENERALE

Au cours de cette thèse, nous nous sommes intéressés aux Perkinsozoa, un groupe de parasites encore peu décrit au sein des Alveolata. Nous avons confirmé que les Perkinsozoa étaient une lignée hors du commun, composée essentiellement de micro-parasites à large spectre d'hôtes dont certains possèdent un potentiel invasif important pouvant représenter une menace écologique, économique et sanitaire à l'échelle mondiale. Bien qu'actuellement composé de quatre clades d'organismes décrits, le séquençage environnemental de différents environnements, e.g. fjords, sources hydrothermales ou sédiments côtiers, a révélé que la diversité génétique de cette lignée était bien plus foisonnante que les quatre clades parasitaires déjà décrits (López-García *et al.*, 2003; Zuendorf *et al.*, 2006; Lepère *et al.*, 2008; Chambouvet *et al.*, 2014). Néanmoins une partie de cette diversité a été révélée sur la base de séquences environnementales qui ne renseignent pas pour autant leur(s) rôle(s) fonctionnel(s) dans les écosystèmes. En effet, peu d'informations sont à ce jour disponibles sur ces organismes putatifs au sein des écosystèmes aquatiques. Nous avons recherché l'occurrence des Perkinsozoa parmi les communautés picoplanctoniques issues des écosystèmes pélagiques échantillonnés lors de l'expédition Malaspina 2010 Circumglobal. Ainsi, de nouveaux clades environnementaux ont été détectés à différentes profondeurs des océans Atlantique, Indien et Pacifique. La présence de ces signatures génétiques dans la fraction ADNr que ARNr permet de confirmer l'existence de clades « actifs⁶ » dans la zone mésopélagique et de clades « hypoactifs⁷ » ou représentés par de l'ADN libre dans la zone bathypélagique. Ces résultats surprenants renforcent la nécessité de mieux comprendre et décrire le groupe des Perkinsozoa dans un contexte global (e.g. mode trophique, spectre d'hôte dans le cas de parasites).

Dans un second temps, nous nous sommes intéressés à un cas particulier de co-infection entre deux parasites déjà décrits, *Perkinsus olseni* et *Perkinsus chesapeaki* (Perkinsozoa, Perkinsidae), infectant les populations de palourdes japonaises au sein du bassin d'Arcachon.

⁶ Organisme-putatif possédant une activité ribosomale. Elle est détectée lorsque le ratio ARNr : ADNr est supérieur à 1. Du fait des faibles abondances des Perkinsozoa dans l'intégralité du jeu de données, les ASVs présents seulement via la détection de leur ARNr sont considérés comme « actifs » car cela peut refléter une activité de transcription (nombre de copies d'ARNr transcrites >> nombre de copies d'ADNr dans le génome) d'un organisme en très faible abondances.

⁷ Organisme-putatif ne possédant pas/plus d'activité ribosomale. Cela peut concerner des organismes sénescents ou en dormance. Elle est déterminée lorsque le ratio ARNr:ADNr est inférieur à 1.

Lors d'un premier échantillonnage réalisé en 2017 nous avons mis en évidence la présence de deux Perkinsidae, *P. olseni* et *P. chesapeaki* au sein d'un même tissu hôte. Nous avons donc mis au point et optimisé une méthode culture-indépendante de qPCR TaqMan en duplex pour la détection simultanée de ces deux parasites au sein des différents tissus de la palourde. Ainsi lors d'une seconde campagne d'échantillonnage en 2018, nous avons pu évaluer simultanément la distribution, la prévalence et l'intensité d'infection de ces deux parasites pour chaque hôte afin d'avoir une approche à l'échelle de la population au sein du bassin d'Arcachon. Nous avons montré que la co-infection par deux parasites est un phénomène complexe impliquant plusieurs facteurs environnementaux, physiologiques (hôte) et écologiques (interaction parasite-parasite et hôte-parasite). Afin de mieux comprendre les stratégies d'infection de ces deux parasites partageant un même hôte, leur génome et leur transcriptome ont été séquencés (analyses en cours).

Cette thèse s'inscrit dans une problématique globale visant à comprendre le rôle fonctionnel de micro-parasites eucaryotes à large spectre d'hôte dans un contexte de changements globaux par des approches *in situ*. Ces travaux apportent de nouvelles connaissances d'abord **à l'échelle des écosystèmes**, en explorant les caractéristiques des parasites déjà décrits ainsi que la diversité environnementale au sein des milieux pélagiques à différents endroits du globe.

PRINCIPAUX RESULTATS

Chapitre 1.1 :

- Les Perkinsozoa (Alveolata) sont un groupe de micro-parasites eucaryotes composé de quatre groupes décrits, dont trois sont marins - les Perkinsidae, les Parviluciferaceae et les Xcellidae - et un est dulcicole - l'agent de la SPI. Au-delà de ces membres déjà décrits, nous avons identifié que de nombreux clusters uniquement formés de séquences environnementales mais aucun n'a été formellement isolé et caractérisé à ce jour.
- Les quatre groupes décrits sont composés de parasites à spectre d'hôte large qui peuvent infecter aussi bien des dinoflagellés, des mollusques, des amphibiens que des poissons. Outre le fait que certains de ces membres possèdent un fort potentiel invasif (e.g. *Perkinsus marinus* et *Perkinsus olseni*), l'infection par ces parasites peut conduire dans certains cas à d'importantes mortalités ayant d'importantes conséquences écologiques (e.g agent de la SPI et Xcellidae) et/ou économiques (e.g Perkinsidae et Xcellidae). Cependant, quelques exceptions à la règle sont à noter comme *Snorkelia prorocentri* ou encore *Xcellia gobii*. Les études s'étant principalement focalisées sur *Perkinsus* spp. et *Parvilucifera* spp., peu d'informations sont disponibles aujourd'hui sur les autres membres décrits.

Chapitre 1.2 :

- Au l'échelle de l'océan globale, le groupe Perkinsozoa forme une lignée composée d'une grande diversité génétique, peu abondant et très localisé dans la colonne d'eau au sein des communautés picoplanctoniques (0.2 - 3 μm). L'analyse de la diversité en ASV (Amplicon Sequencing Variant) a permis de déterminer six groupes majeurs (clusters A à F) dont certains sont proches de groupes déjà décrits – e.g le cluster F partage 88 à 94 % d'identité avec les séquences des genres *Snorkelia*, *Dinovorax* et *Tuberlatum* ; les clusters B et C se rapprochent des parasites de poissons du genre *Xcellia* et *Gadixcellia* ou encore le cluster D se retrouve en position basale des Perkinsidae.
- Le cluster A domine la diversité avec 76 ASVs (109 ASVs au total) ainsi que les abondances, avec 40 % des reads ADN et 64 % des reads ARN, au sein des Perkinsozoa. Cependant ce cluster reste totalement énigmatique car il n'est affilié à aucune séquence disponible dans les bases de données publiques.
- Cette diversité est absente de la zone photique (surface et maximum de chlorophylle (DCM)) mais se retrouve plus en profondeur dans la zone mésopélagique (200 – 1 000 m), où les clusters A, B et C sont les plus actifs, et dans la zone bathypélagique (1 000 – 4 000 m), où les clusters D, E et F sont hypoactifs voire complètement inactifs car uniquement représentés par leurs séquences issues de la fraction ADNr.

Cette thèse apporte également de nouvelles connaissances **à l'échelle des populations**, par l'étude de la co-infection entre deux espèces de parasites et leur hôte *R. philippinarum*, la palourde japonaise. Ces résultats confirment l'ubiquité des infections multiples par différentes espèces de *Perkinsus* spp. et révèlent la complexité de leur distribution *in situ* et des interactions de facteurs environnementaux et biologiques qui les contrôlent.

PRINCIPAUX RESULTATS**Chapitre 2.1 :**

- La qPCR en duplex est un outil moléculaire fiable sans étape de culture pour la détection et la quantification simultanées de deux parasites, *P. olseni* et *P. chesapeaki*, vivant en sympatrie au sein de différents organes chez la palourde japonaise, *R. philippinarum*.
- La présence d'inhibiteurs de PCR dans les différents organes de la palourde japonaise ont conduit à une optimisation des seuils de concentration et des limites de détection.

- Les résultats obtenus par qPCR en duplex ont été corrélés aux résultats issus de méthode classique RFTM recommandée par l'O.I.E. Ainsi la qPCR en duplex se révèle être une méthodologie particulièrement efficace pour la quantification des intensités d'infection moyennes à fortes.

Chapitre 2.2 :

- En investiguant les différents compartiments tissulaires chez la palourde japonaise, nous avons mis en évidence que la co-infection est largement répandue au sein du bassin d'Arcachon allant de 12 à 36 % de prévalence.

- Les prévalences et les intensités d'infection de la mono-infection à *P. olseni* dominent largement les palourdes échantillonnées alors que la mono-infection à *P. chesapeaki* y est extrêmement rare et localisée.

- Les variations observées dans la distribution des infections globales à *Perkinsus* spp. entre les différentes stations peuvent être expliquées par leur caractéristiques abiotiques (e.g. salinité, sédiments, influence de l'Atlantique). En effet, les intensités d'infection apparaissent plus importantes dans les zones sous influence océanique où les palourdes sont les plus âgées (taille de la coquille plus importante) que dans les zones sous influence continentale.

- Bien que la prévalence des co-infections soit ponctuelle, elle n'est détectée généralement que dans un ou deux organes au sein d'un individu (e.g. la branchie ou le muscle adducteur ou encore le manteau). Cependant au sein de ces organes co-infectés, la charge parasitaire est majoritairement dominée par *P. olseni*.

- Globalement, la faible occurrence de la mono-infection à *P. chesapeaki* ainsi que la récurrence de la co-détection des deux espèces de parasites laissent supposer que *P. olseni* pourrait avoir un rôle décisif de facilitation des infections secondaires à *P. chesapeaki*. Cependant cette relation pourrait être dynamique et pourrait conduire vers une compétition (directe ou indirecte) entre les deux parasites lorsque ces parasites sont co-localisés dans les mêmes tissus et lorsque la charge parasitaire de *P. olseni* devient trop importante.

Chapitre 2.3 :

- Les génomes et les transcriptomes de *P. olseni* et *P. chesapeaki* ont été séquencés suite à la mise en place de cultures monoclonales à partir de souches isolées de palourdes japonaises échantillonnées dans le bassin d'Arcachon lors de la campagne d'échantillonnage 2018.

1. La co-infection entre deux parasites à large spectre d'hôtes et potentiellement invasifs

1.1. Les méthodologies moléculaires essentielles à la caractérisation des co-infections cryptiques

Le parasite *P. olsenii* est responsable de nombreux épisodes de mortalité dans les populations de palourdes entraînant d'importantes pertes économiques dans la filière conchylicole principalement en Asie et en Europe (e.g Choi and Park, 2005, 2010; Pretto *et al.*, 2014). Ces événements lui ont valu d'être répertorié par l'O.I.E dans la liste des « Maladies, infections et infestations » étant à risque pour les échanges commerciaux et internationaux (Organisation mondiale de la santé animale (O.I.E, 2019b). Ainsi, l'O.I.E recommande aujourd'hui certains protocoles de diagnostic ciblant la Perkinsose comme la méthode de culture RFTM, l'histologie et la PCR (O.I.E, 2019b) lors de mortalités anormales dans les populations de bivalves exploitées. Parallèlement, de récentes études ont révélé que plusieurs espèces appartenant au genre *Perkinsus* pouvaient être fréquemment détectées au sein d'un même hôte à travers le monde - e.g au Japon (Takahashi *et al.*, 2009), aux Etats-Unis (Reece *et al.*, 2008), au Brésil (da Silva *et al.*, 2014), en Espagne (Ramilo *et al.*, 2016) ou en France (Arzul *et al.*, 2012). Bien que l'infection par de multiples parasites appartenant aux Perkinsidae semblent être plutôt la règle que l'exception, ces co-infections sont paradoxalement peu étudiées. Or, ces infections multiples peuvent radicalement influencer le devenir de certains parasites, leur conséquences sur la « fitness » de leurs hôtes et le développement de maladies (Bordes and Morand, 2011; Bass *et al.*, 2019).

Dans cette thèse, en prenant comme site modèle le bassin d'Arcachon, nous avons montré que la co-infection entre *P. olsenii* et *P. chesapeaki* était un phénomène récurrent qui a pu passer inaperçu par l'usage des techniques culture-dépendantes et RFTM (Chapitre 2.1 et 2.2). Grâce à la méthodologie de qPCR en duplex optimisée pour chacun des tissus de la palourde japonaise, il est maintenant possible de déterminer la prévalence et l'intensité d'infection *in situ* de chacun des deux parasites au sein d'un même tissu (Chapitre 2.1 et 2.2). Cette technique a permis la détection simultanée de ces deux parasites dans 12 à 36 % des individus prélevés au sein de quatre stations du bassin d'Arcachon (Chapitre 2.2). Qui plus est cette co-détection n'était pas toujours localisée dans la branchie, qui est généralement l'organe ciblé pour le diagnostic de la Perkinsose à l'échelle de l'individu comme recommandé par l' O.I.E. (O.I.E, 2019b; Dang *et*

al., 2013). En effet, la détection de ces infections, uniquement dans la branchie, sous-estime la distribution globale de la co-infection (prévalence dans la branchie entre 6 % et 24 % sur seulement trois stations du bassin). L'analyse de l'intégralité du corps des palourdes est donc à privilégier lorsque la dynamique d'infection et la prolifération des parasites ne sont pas connues.

Dans un champ d'application lié à la pathologie vétérinaire, cette méthode pourrait être utilisée parallèlement à la méthode de culture RFTM qui identifie uniquement les organismes appartenant au genre *Perkinsus* sans discriminer l'espèce. Au contraire, la méthodologie en duplex qPCR permet simultanément de quantifier le nombre de copies d'opéron ribosomal des deux espèces de parasites. La comparaison entre ces deux méthodologies a permis de révéler quelques décalages au niveau des faibles intensités d'infection. Cependant sur les infections moyennes à fortes comme celles déterminées dans le bassin d'Arcachon, l'application de cette méthodologie s'est révélée pertinente. Ainsi, bien que cette méthodologie soit prometteuse, il est impératif de prendre en compte ses limites techniques: **1)** nous ne savons pas quels stades du cycle de vie avec des ploïdies variables sont ainsi quantifiés (e.g. trophozoïtes ou schizontes); **2)** le nombre de copies de l'opéron ribosomal est inconnu pour chaque espèce de parasite; **3)** cette méthode de détection demande d'identifier en amont les parasites déjà présents au sein des hôtes afin de développer des amorces et des sondes spécifiques pour leur quantification. Dans notre étude, la mise en culture lors de la campagne en 2017 et 2018 nous a permis d'isoler ces deux espèces de parasite et d'obtenir suffisamment de matériel génétique pour optimiser la méthode. De plus, dans le cas de co-culture entre deux espèces de parasites, l'une d'elles peut prendre le dessus en termes d'abondance et /ou inhiber la seconde. Ainsi des infections cryptiques pourraient passer inaperçues en culture et induire un biais dans l'estimation de la diversité des infections à *Perkinsus*. Une autre solution plus fastidieuse aurait été de réaliser une première détection par amplification PCR avec des amorces eucaryotes Perkinsidae ou Perkinsozoa générales dans les individus échantillonnés et de cloner ces produits afin d'avoir une représentation significative de leur diversité au sein des tissus hôtes. Cependant toutes ces approches sont basées sur des *a priori*. Ainsi, une approche plus descriptive basée sur le séquençage intégral des communautés symbiotiques au sein de l'hôte serait idéale afin de connaître leur diversité globale à différents endroits du bassin. Ceci permettrait le développement d'une nouvelle méthode de qPCR en multiplexe incluant les taxa susceptibles d'induire une maladie selon la littérature disponible mais aussi ceux dont les abondances ou les co-occurrences, déterminées par des outils de NGS, pourraient être significatives dans la mise

en place d'un pathobiome (Bass *et al.* 2019). D'un point de vue écologique, connaître cette diversité symbiotique et ses interactions au sein d'un hôte est essentiel pour déterminer le rôle et le devenir des infections au sein des écosystèmes. Une méthode de multiplexage pourrait permettre de suivre les dynamiques parasitaires *in situ* au sein de différentes populations d'hôtes appartenant à différents habitats.

1.2. Préférence d'hôtes : paramètre déterminant dans la co-infection

Les parasites du genre *Perkinsus* (syn. Perkinsidae) sont décrits comme étant des parasites à large spectre d'hôte cependant, toutes les infections ne se valent pas. Cette observation a été réalisée lors de l'analyse de l'ensemble de la littérature sur les infections par le genre *Perkinsus* (Chapitre 1.1). Nous pouvons voir que malgré une détection dans un grand nombre d'espèces les prévalences peuvent être très variables : *P. chesapeaki* a des prévalences plus faibles chez les Ostreidae (< 20 %) que chez des espèces de Myidae, Tellinidae et Veneridae (> 20 %) ; *P. olseni* est peu prévalent chez les Ostreidae (< 20 %) contrairement à ce que l'on observe chez les Pteridae ou les Veneridae. Ces différences ont déjà été relevées à la suite d'études *in situ*, Reece *et al.* ont confirmé en 2008 que malgré les co-infections entre *P. marinus* et *P. chesapeaki* au sein de mêmes hôtes, *P. marinus* serait un parasite ayant pour préférence d'infection certaines espèces d'huîtres alors que *P. chesapeaki* serait plus à même d'infecter des espèces comme *Mya arenaria*, *Mercenaria mercenaria* ou *Tagelus plebeius*. Cette question se pose pour les co-infections entre *P. olseni* et *P. chesapeaki* observées chez *R. philippinarum* en Espagne (Ramilo *et al.*, 2016) et en France (Arzul *et al.*, 2012). Dans cette thèse, nous nous retrouvons confrontés à deux infections très différentes entre *P. olseni* et *P. chesapeaki* chez la palourde japonaise. Dans le cas de *P. olseni*, nous avons observé des prévalences (>90% dans le bassin d'Arcachon) et un schéma de prolifération classiquement décrit dans la littérature avec une infection principale dans les branchies puis dans les organes secondaires comme le manteau et la glande digestive et enfin dans le muscle adducteur et le pied (Wang *et al.*, 2018). Pour *P. chesapeaki*, l'infection est ponctuelle voire accidentelle comme décrit par Arzul *et al.* en 2012 et Ramilo *et al.* en 2016. La faible prévalence de mono-infection à *P. chesapeaki* et ses faibles intensités d'infection lors de co-infection avec *P. olseni* laissent supposer que *R. philippinarum* ne fait pas partie de ses hôtes préférentiels. Ce parasite aurait donc besoin d'une primo-

infection, ici avec *P. olseni*, qui faciliterait son entrée dans l'hôte. La question du spectre d'hôte est donc une donnée essentielle pour comprendre les co-infections. L'infection d'un hôte « non-préférentiel » pourrait être basée sur des processus chimiques et physiologiques communs à plusieurs hôtes apparentés comme observé chez *Parvilucifera sinerae* (Garcés *et al.* 2013b). Ces processus, hypothétiquement plésiomorphes chez des espèces d'hôtes proches, pourraient faciliter les infections multiples entre plusieurs espèces du genre *Perkinsus*.

Il est actuellement admis que le parasite *P. olseni* a potentiellement été introduit avec son hôte *R. philippinarum* en Europe (Vilas *et al.*, 2011; Cordero *et al.*, 2017), depuis il s'est répandu le long de la côte Atlantique avec une faible diversité génétique (Vilas *et al.*, 2011). Cependant, la présence de *P. chesapeaki* pose plus de questions car ce parasite aurait été introduit en Europe à partir des États-Unis via son/ses vecteur(s) d'introduction en Europe, *Mya arenaria* ou *Mercenaria mercenaria* (Arzul *et al.*, 2012). En 1861 et 1863, le bassin d'Arcachon est le site privilégié pour tester l'introduction de *Mercenaria mercenaria* mais a finalement échoué (Ruckebusch, 1949). Cette introduction à court terme pourrait être l'origine de la propagation de certains génotypes de *P. chesapeaki* dans cette aire. Ainsi, le dernier aurait pu se disséminer à des espèces indigènes comme *Mya arenaria* déjà présentes dans le bassin (Blanchet, 2004). Mais, les séquences ITS de *P. chesapeaki* issues de cultures isolées du bassin d'Arcachon partagent 98 % de similitude avec les séquences européennes alors que ce sous-groupe ne partage que 96 % de similitude avec les séquences venant des États-Unis et d'Australie (Reece *et al.*, 2017). La phylogénie de *P. chesapeaki* montre une nette dichotomie entre ces deux sous-groupes qui interroge sur l'origine et l'évolution de ce parasite en Europe. Nous pouvons émettre l'hypothèse que si *P. chesapeaki* a été introduit en Europe à partir de souches Américaines alors ce parasite aurait pu opérer un glissement d'hôtes dans son nouvel environnement et ainsi de profiter d'une primo-infection par *P. olseni* pour se maintenir au sein d'un hôte réservoir non préférentiel. Ces préférences d'hôtes pourraient être vérifiées dans le cadre d'expérimentation d'infection *in vitro* du point de vue de : **1) l'hôte**, par l'évaluation de profils physiologiques et immunitaires chez différentes espèces (présence de lésion par histologie, évaluation du stress oxydatif et de l'activité hématocytaires) ; et **2) des parasites**, par l'évaluation des prévalences, des intensités d'infections, du taux de multiplication et de dispersion, la caractérisation et la quantification de composés protéiques extracellulaires (ECP) (La Peyre *et al.*, 1993; La Peyre *et al.*, 1995; McLaughlin *et al.*, 2000).

Il est aujourd'hui compliqué de comprendre exactement la présence de *P. chesapeaki* au sein de ces populations de palourdes japonaises. Dans cette étude, l'intégration d'autres espèces d'hôtes potentiels vivant en sympatrie avec *R. philippinarum* aurait pu être un élément de réponse pour confirmer ou infirmer les préférences de chacun des deux parasites. Sur la base de la méthode moléculaire développée au Chapitre 1.1, il aurait été possible de comparer les prévalences et les intensités d'infection entre différentes espèces par exemple en prenant les coques, *Cerastoderma edule*, qui se trouvent être fortement infectées par *P. chesapeaki* en Espagne (Carrasco *et al.*, 2014). La mise en évidence de cette vie en sympatrie entre *P. olseni* et *P. chesapeaki* a conduit au séquençage de leur génome et de leur transcriptome (Chapitre 2.3). Ces données pourront nous éclairer sur les différences dans le métabolisme et la virulence de ces deux parasites par l'identification des polymorphismes **1) des facteurs de virulence** (La Peyre *et al.*, 1995; Tall *et al.*, 1999), **2) des facteurs physiologiques**, par exemple en explorant les gènes exprimés lors la défense contre le système immunitaire hôte (e.g Superoxyde Dismutase) (Fernández-Boo *et al.*, 2014, 2015), **3) des facteurs métaboliques** impliquant les voies de synthèse (acides aminés, lipides) ou les voies de prolifération/mort cellulaire (Hasanuzzaman *et al.*, 2016).

Enfin, la méthode que nous avons mise au point permet de donner l'état de l'infection à un instant T mais ne renseigne pas sur la dynamique des deux parasites dans les tissus. Pour aller plus loin dans ces interactions intra-hôtes, il serait intéressant de suivre l'activité métabolique parasitaire lors d'une co-infection au sein de différents hôtes. Des outils moléculaires, comme la RT-qPCR, pourraient être optimisés pour quantifier l'expression de plusieurs gènes discriminants liés à la virulence, à la division cellulaire ou à l'apoptose. Cela permettrait de déterminer si ces relations de co-infection impliquent de la compétition (directe ou indirecte), de la facilitation ou de la neutralité entre les parasites. Ces profils d'expression pourraient être reliés à des paramètres physiologiques et immunitaires de chacune des espèces d'hôtes présentes dans un même habitat afin de mieux comprendre la spécificité du spectre d'hôte au sein du genre *Perkinsus*.

1.3. La co-infection sous l'influence de plusieurs paramètres biotiques et abiotiques

Aujourd'hui nous savons que les conditions environnementales affectent la répartition des espèces appartenant au genre *Perkinsus*. Par exemple, *P. olseni* se retrouve à de faibles intensités d'infection dans les zones à faible salinité (Dang *et al.*, 2010; Binias *et al.*, 2014; Waki and Yoshinaga, 2015). De même la prolifération et la dissémination de *P. marinus* augmentent avec les fortes températures (Ford, 1996; Ford and Smolowitz, 2007; Gignoux-Wolfsohn *et al.*, 2020). Ces paramètres peuvent aussi influencer le développement de certains stades de leur cycle de vie. Par exemple, pour *P. olseni*, la baisse du pH, de l'oxygénation ainsi que l'important relargage de nutriments stimulent la transformation des trophozoïtes en hypnospores (probable stade de résistance) (Casas and La Peyre, 2013). Les conditions de zoosporulation chez *P. chesapeaki* et *P. olseni*, ont été observées *in vitro* à des salinités de 20 psu et 15 et 35 psu respectivement (McLaughlin *et al.*, 2000; Umeda *et al.*, 2013). L'optimal de survie des hypnospores de *P. olseni* se situe à une salinité comprise entre 25 et 35 psu (Casas *et al.*, 2002; Umeda *et al.*, 2013). Sachant que les conditions environnementales façonnent le cycle de vie de ces parasites, la distribution et les profils de co-infection observés dans le bassin d'Arcachon au sein de *R. philippinarum* (Chapitre 2.2) pourraient être le résultat complexe des interactions entre les paramètres abiotiques de l'habitat, l'état physiologique de l'hôte, les caractéristiques du spectre d'hôte et les possibles interactions parasite-parasite. Cependant, les raisons exactes de l'existence de ces profils de co-infection (localisés dans la branchie, la glande digestive ou le muscle adducteur) restent encore floues. Nous n'avons pas formellement identifié les facteurs pouvant expliquer cette distribution à l'échelle des stations que nous avons échantillonnées en 2018. Pour le moment, le lien entre stades du cycle de vie de *P. chesapeaki* et spécificité de l'organe « porte d'entrée » n'a pas encore été élucidé. Aucune étude dans le sédiment (présence des formes de résistance - hypnospores) ni dans la colonne d'eau (présence de stades libres flagellés - zoospores) n'a été réalisée pour détecter et isoler ces potentiels stades.

A l'échelle populationnelle, l'étude que nous avons menée sur la co-infection apporte une bonne représentation des prévalences et des intensités d'infection pour *P. olseni* et *P. chesapeaki* au sein des populations de palourdes japonaises du bassin d'Arcachon. Cependant, d'autres parasites sont aussi présents dans cette aire, dont certains sont responsables de maladies comme la BMD (virus potentiel - Dang *et al.*, 2008; Pierron *et al.*, 2019), la BRD (infection par *Vibrio tapetis* - Lassalle *et al.*, 2007; de Montaudouin *et al.*, 2010) ou encore la trématodose

(infestation par des trématodes - Dang *et al.*, 2009; Paul-Pont *et al.*, 2010). Au cours de notre étude, nous avons procédé à une détection visuelle de la BMD et de la BRD même si les prévalences relevées restent des variables secondaires car l'effort d'investigation est largement inférieur à celui mis en place pour l'étude de *Perkinsus* spp. Bien que la détection de la BMD puisse se faire visuellement comme recommandé par Dang *et al.* en 2008, la détection de la BRD aurait dû être réalisée par PCR afin de confirmer sa prévalence (Park *et al.*, 2006).

A l'issu de cette thèse nous ne possédons qu'une vision partielle des infections chez *R. philippinarum* dans le bassin (Figure 1). Une étude représentative de l'écosystème devrait être privilégiée pour comprendre tous les facteurs biotiques et abiotiques influençant les infections par ces deux parasites. Dans un premier temps, il faudrait intégrer la diversité des communautés symbiotiques résidant au sein d'un hôte afin de mieux comprendre les assemblages de micro-organismes (coexistence, exclusion, prolifération) qui influent sur la fitness et sur l'apparition de potentielle maladie (Bass *et al.*, 2019). Dans un second temps, il faudrait intégrer les hôtes potentiels pour chacun des parasites impliqués dans cette infection multiple. Par exemple, nous suspectons *P. chesapeaki* de posséder un hôte préférentiel différent de la palourde japonaise mais celui-ci n'a pas été identifié à ce jour. Il est donc plus difficile de comprendre le mode de transmission et de dispersion de ce parasite chez *R. philippinarum*. Aujourd'hui, le système « un hôte et ses parasites » devrait s'élargir à une approche plus intégrée de son environnement. Il est essentiel d'établir des réseaux d'infection permettant de mieux comprendre la dynamique des parasites dans un écosystème et de prévenir leur invasion dans de nouveaux habitats. Une bonne connaissance de leur environnement *in situ*, sur le plan biotique et abiotique, est nécessaire pour traiter ce genre de problématique. Retracer ces réseaux d'interactions (incluant les hôtes préférentiels, paraténiques et réservoirs) permettrait d'endiguer la dispersion de ce type de parasites au fort potentiel invasif.

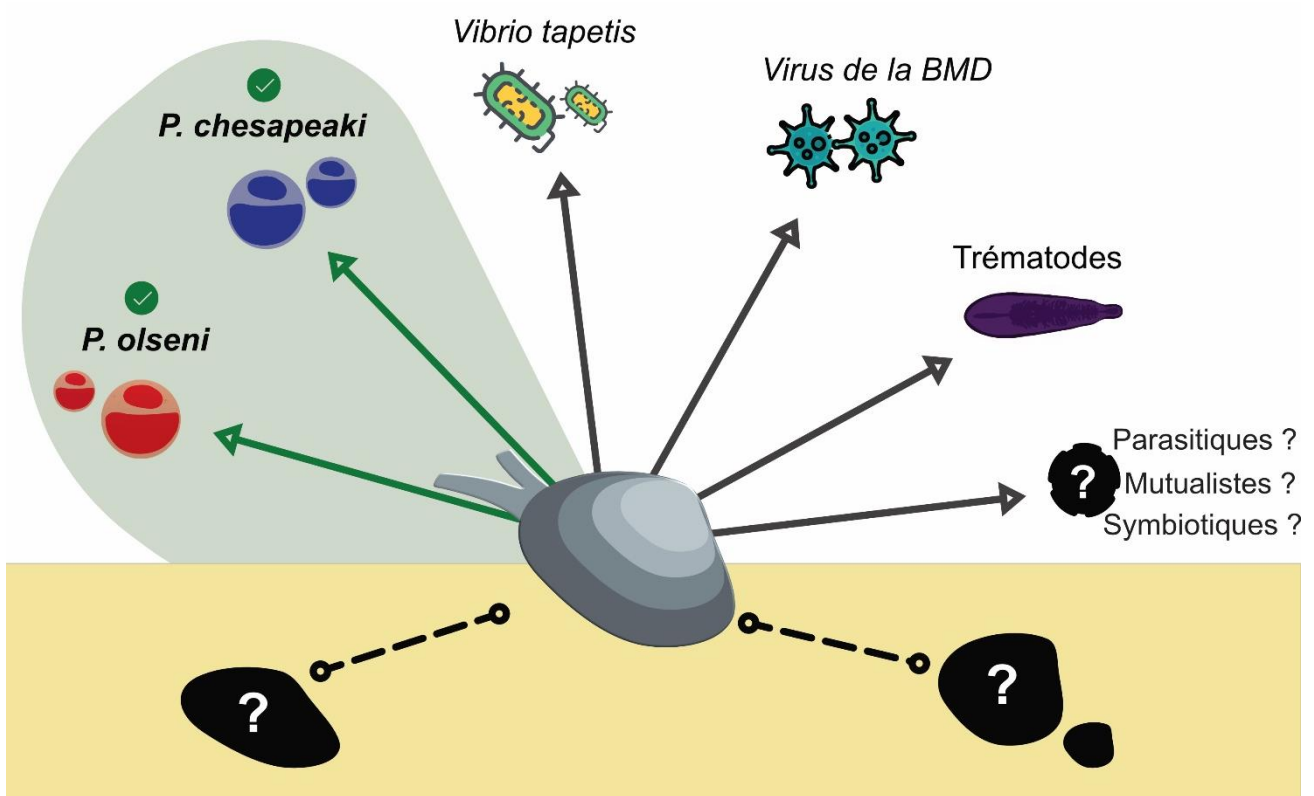


Figure 1. Approche intégrative du modèle hôtes-parasites : *R. philippinarum* – *P. olseni* – *P. chesapeakei* comprenant la faune benthique en sympatrie et les communautés de micro-organismes symbiotiques. Les flèches vertes représentent les interactions étudiées au cours de cette thèse.

2. Les Perkinsozoa : une diversité riche mais énigmatique

Aujourd’hui, les Perkinsozoa sont composés de quatre clades principaux de parasites généralistes décrits et d’une large part de diversité environnementale présente en eau douce et en milieu marin. Certains membres comme l’agent responsable de la SPI peuvent être pathogènes et participer, en synergie avec d’autres parasites, au déclin d’espèces clé de voûte, ingénieuses et en voie de disparition (Annexe 1: Chambouvet *et al.*, 2020). Ils peuvent aussi entraîner d’importantes pertes économiques et d’importantes modifications structurales dans les écosystèmes : **1)** les **Xcellidae** sont potentiellement responsables de mortalités chez les juvéniles de certains poissons exploités (Morrison *et al.*, 1982) ; **2)** l’**agent de la SPI** participant au déclin global des populations d’amphibiens (Annexe 1: Chambouvet *et al.*, 2020) ; **3)** certaines espèces chez les **Perkinsidae** ont décimé des récifs d’huîtres en baie de Chesapeake

impliquant une baisse du contrôle top-down de ces filtreurs sur le phytoplancton (Andrews, 1996) ; et **4**) les **Parviluciferaceae** semblent aussi jouer un rôle fonctionnel essentiel dans la régulation d'efflorescence de dinoflagellés en Europe (Alacid *et al.*, 2017) comme cela a été précédemment montré pour *Amoebophrya* sp. (Chambouvet *et al.*, 2008).

A ce jour, la majorité des Perkinsozoa ne sont décrits que par leur signature génétique (Bråte *et al.*, 2010; Chambouvet *et al.*, 2014). Ainsi la morphologie, le mode trophique ou encore le cycle de vie de ces organismes restent très énigmatiques. Bien que la diversité des Perkinsozoa ait tout d'abord été décrite comme foisonnante en milieu lacustre (e.g. Lepère *et al.*, 2008; Mangot *et al.*, 2011) leur conférant un rôle fonctionnel saisonnier majeur dans ces écosystèmes, Chambouvet *et al.* en 2014 montrèrent que la majorité des séquences environnementales marines seraient majoritairement issues du compartiment benthique et très peu représentées dans de la colonne d'eau (surface et DCM).

Au cours de cette thèse, nous avons mis en avant la diversité des Perkinsozoa dans les écosystèmes pélagiques au sein de l'océan Atlantique, Pacifique et Indien échantillonnés lors de la campagne Malaspina 2010 Circumglobal (Duarte, 2015) (Chapitre 1.2). Les six clades détectés en zones mésopélagique et bathypélagique nous montrent une diversité encore inconnue au sein des communautés picoeucaryotes (0.2 - 3µm). Cette diversité génétique a été retrouvée aussi bien dans la fraction ADNr que ARNr, montrant que ces clades sont ribosomalement actifs et pourraient jouer un rôle fonctionnel et écologique encore indéterminé dans ces écosystèmes. Bien que tous les membres décrits des Perkinsozoa soient des parasites, il n'est pas possible de trancher définitivement en faveur d'un mode de vie parasitaire concernant les clades identifiés. Ainsi, la présence de cette diversité parmi les picoeucaryotes interroge sur ce que pourrait être ces organismes putatifs. Plusieurs hypothèses sont envisageables : **1**) cette fraction de taille pourrait correspondre à des stades de vie flagellés (zoospores, 1-2µm) comme décrits chez *Perkinsus* spp. et *Parvilucifera* spp. Cela pourrait indiquer l'existence de nouveaux organismes parasites encore non décrits infectant des hôtes présents dans la zone mésopélagique ; **2**) ce sont de petits hétérotrophes libres. En effet, en zone mésopélagique les communautés picoeucaryotes sont composées majoritairement d'hétérotrophe libres, de parasites ou de mixotrophes dont les plus dominants sont les Ciliés, les MALV, les Dinoflagellés, les Labyrinthulomycètes et les Cercozoaires (Edgcomb, 2016; Giner *et al.*, 2020); **3**) ces séquences sont issues d'un biais d'échantillonnage lié à la filtration. Ces séquences pourraient être affiliées à des organismes, hétérotrophes libres ou symbiotiques, appartenant à une fraction de taille supérieure (2 – 20 µm) qui auraient pu être cassés lors de la

filtration. Concernant les clusters A, B et C, il est peu probable que leur diversité soit associée à celle d'organismes phytoplanctoniques comme des dinoflagellés infectés par *Parvilucifera* spp. car leurs signatures génétiques sont totalement absentes de la zone photique bien qu'échantillonnée à 146 stations de surface et DCM durant la campagne océanographique Malaspina 2010 Circumglobal. Si association il y a, elle se produirait exclusivement en interaction des organismes de petites ou grandes tailles de la zone mésopélagique. L'accès aux séquences ASVs de l'ARNr et de l'ADNr du nanoplancton (3 - 20 μm) pourrait permettre de clarifier la provenance de ces séquences et une analyse statistique de « co-occurrence » pourrait permettre d'identifier les potentiels couples associés (Lima-Mendez *et al.*, 2015). Enfin, pour confirmer une ou plusieurs de ces hypothèses, il faudra procéder au développement de sondes spécifiques à ces différents clusters actives couplées à des colorants cellulaires ciblant les lysosomes (détermination de l'hétérotrophie (Wideman *et al.*, 2020) par le transport d'enzymes digestives comme la bêta-D-Glucosaminidase (Sintes and Del Giorgio, 2010)) ou des colorants nucléaires (viabilité des potentielles espèces hôtes - voir méthodologie Annexe 2: Chambouvet *et al.* 2019). Il sera donc possible de localiser ces organismes dans la colonne d'eau par microscopie FISH sur les filtres échantillonnés lors de la campagne Malaspina 2010

De nombreuses détections environnementales des Perkinsozoa ont été relevées en milieu marin que ce soit lors d'études globales des communautés de micro-organismes (De Vargas *et al.*, 2015) que lors d'études spécifiques au sein des écosystèmes côtiers (Chambouvet *et al.*, 2014). Il aurait été intéressant de pouvoir récupérer ces séquences des différentes études environnementales menées sur les sédiments, les fjords anoxiques ou encore les champs hydrothermaux afin de les comparer avec la diversité retrouvée en zone pélagique. Bien souvent, les techniques de séquençage (pyroséquençage 454 et séquençage Illumina), les régions de l'ARNr séquencées (V9 ou V4 (Pawlowski *et al.*, 2012)) et les méthodes d'analyses (Swarm (Mahé *et al.*, 2015) et DADA2 (Callahan *et al.*, 2016)) divergent et peuvent rendre ces études difficilement comparables. Par exemple, la méthode de regroupement taxonomique par OTUs (Operational Taxonomic Unit) et par ASVs (Amplicon Sequence Variant) montrent des disparités sur l'évaluation de la diversité des communautés de protistes (Hu and Caron, 2018). Un consensus au sein des méthodes et des marqueurs moléculaires utilisés (Pawlowski *et al.*, 2012) pourrait contribuer à améliorer la détection globale de phyla comme les Perkinsozoa et de comprendre le plus justement possible la diversité globale de ces organismes à grande échelle.

Aujourd'hui, certains exemples de détection environnementale ont abouti à l'identification et à la caractérisation d'organismes potentiellement parasites comme cela a été le cas pour *Haplosporidium diporae*, anciennement « Haplosporidian clade C » (Hartikainen *et al.*, 2014; Winters and Faisal, 2014; Bass *et al.*, 2015), et pour le clade NCLC1 (novel chytrid-like-clade-1) (Annexe 2 : Chambouvet *et al.*, 2019). Ainsi, il serait intéressant d'approfondir et de caractériser les clusters identifiés au sein des communautés de picoeucaryotes à travers l'océan global (Chapitre 1.2). Pour cela des méthodes de microscopie couplées à des analyses bio-informatiques de co-occurrence permettraient de faire émerger de potentiels réseaux d'interactions entre protistes, zooplancton ou grands métazoaires (Lima-Mendez *et al.*, 2015). Ces réseaux seraient très utiles dans le cas de taxa parasites car ils permettraient la quantification des parasites et des hôtes et ainsi d'apporter de nouvelles pistes sur l'influence des microbiomes sur les infections.

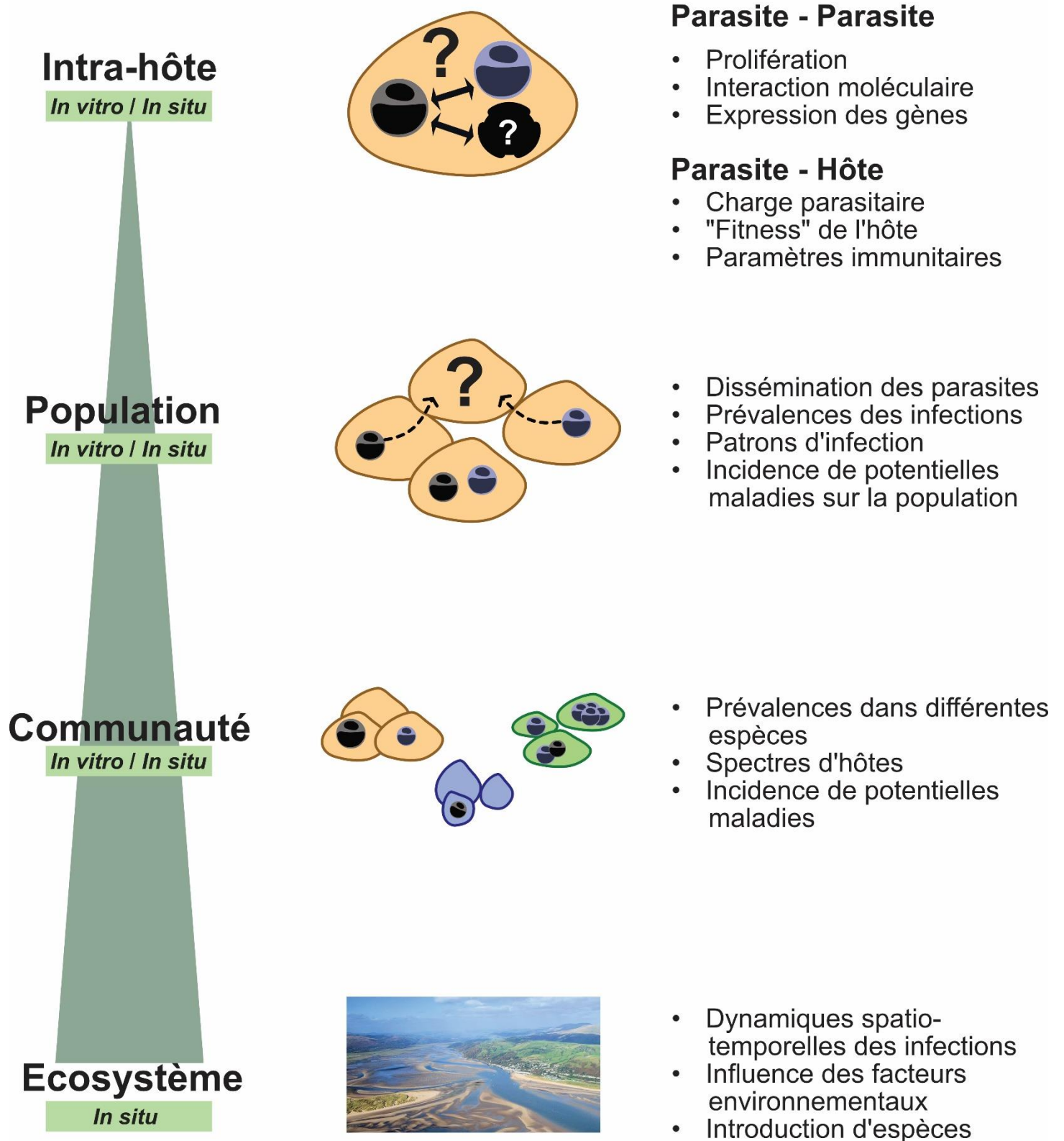


Figure 2. Les différentes échelles pour appréhender l'écologie globale d'un ou de plusieurs parasites. Les paramètres pouvant être mesurés à chaque niveau sont spécifiés à droite des illustrations. Les encarts verts déterminent si les études peuvent être conduites *in situ* et/ou *in vitro*.

PERSPECTIVES

Au cours de cette thèse, nous nous sommes d'abord intéressés à comprendre la diversité génétique des Perkinsozoa à l'échelle globale. Nous nous sommes ensuite focalisés sur le décodage des phénomènes de co-infection à l'échelle populationnelle. Afin de comprendre le lien entre diversité environnementale et rôle écologique au sein du groupe des Perkinsozoa, il serait intéressant de coupler plusieurs échelles d'étude (Figure 2) :

- 1) **L'échelle intra-hôte**, qui permettrait de résoudre, *in vitro* et *in situ*, les mécanismes d'interaction entre plusieurs parasites au sein d'un hôte par l'estimation de leur taux de multiplication, d'expression de certains gènes de virulence ou de prolifération ou encore de leurs interactions moléculaires. Concernant les deux parasites *P. olseni* et *P. chesapeaki*, une approche de génomique comparative a été privilégiée (Chapitre 2.3) avec le séquençage de deux génomes et deux transcriptomes. Aujourd'hui de nouveaux génomes sont disponibles pour ces deux parasites provenant d'hôtes très différents (e.g *Anadara trapezia*, *Haliotis rubra*, *Austrovenus stutchburyi*) (Bogema *et al.*, 2020), il serait donc intéressant de réaliser une étude comparative de ces génomes afin de comprendre si l'évolution de la virulence et des métabolismes chez ces parasites. L'accès à ces génomes provenant d'Asie, d'Australie et de France apporterait ainsi une résolution de la plasticité observée sur leurs mécanismes d'infection.
- 2) **L'échelle populationnelle**, afin d'étudier la dynamique d'infection au sein d'une même population hôte comme nous l'avons fait au cours des Chapitres 2.1 et 2.2. Une approche générale, sans *a priori* sur les communautés de micro-organismes, pourraient permettre de comprendre certains patrons de mono-infection et de co-infection au sein d'une population d'hôte. Par l'intermédiaire du séquençage haut-débit, nous pourrions relier ces cortèges de micro-organismes à l'occurrence et aux interactions de certains parasites étudiés. Ces assemblages permettraient de mieux appréhender l'écologie de ces parasites et l'occurrence de certaines maladies.
- 3) **L'échelle communautaire**, qui permettrait de comprendre pleinement la circulation des parasites au sein de différentes espèces d'hôtes vivant en sympatrie dans un même écosystème. Cela permet d'appréhender les espèces paraténiques ou réservoirs du

spectre d'hôte et d'établir ainsi leurs préférences respectives. Il faudrait alors appliquer les mêmes méthodologies moléculaires qu'à l'échelle populationnelle (PCR, qPCR, NGS) et les répercuter sur différentes espèces d'hôtes potentiels. De plus, il conviendrait de procéder à ces détections moléculaires dans des compartiments abiotiques pouvant servir de réservoir pour ces parasites, comme par exemple les sédiments en milieu marin qui recueillent une grande diversité de forme de résistances produites par certains protistes.

- 4) L'échelle écosystémique**, afin d'intégrer les informations acquises à l'échelle des communautés dans un cadre spatial et temporel (e.g cycle tidal, saisonnier, annuel) plus large. Il sera possible de comprendre l'influence des paramètres environnementaux sur la dynamique des parasites au cours du temps. Pour cela il serait intéressant de pouvoir échantillonner des écosystèmes très contrastés avec les mêmes procédures d'échantillonnage. Les comparaisons inter-écosystèmes serviraient à évaluer l'écologie et la plasticité de ces parasites à différents endroits du globe.

Ces différents niveaux d'étude pourraient être une clé de compréhension des mécanismes évolutifs chez les Perkinsozoa. En effet, leur combinaison permettrait d'appréhender pleinement leur rôle au sein des réseaux trophiques aquatiques. Dans le cas où certains de ces organismes seraient des hétérotrophes libres, ils devront être impérativement caractérisés afin d'identifier leur rôle écologique à différentes échelles (e.g participation aux flux biogéochimiques ou aux flux d'énergie dans les réseaux trophiques). Il s'agirait ainsi de la première identification d'hétérotrophes libres dans ce groupe, ce qui serait intéressant du point de vue de l'évolution de la photoautotrophie et de l'hétérotrophie au sein des Alveolata.

Pour conclure, les membres décrits dans les Perkinsozoa sont très souvent associés à des mortalités dans les milieux aquatiques et leur présence est souvent perçue comme négative surtout en ce qui concerne les Perkinsidae, les Xcellidae ou l'agent de la SPI. Cependant, sachant qu'une part de la diversité des Perkinsozoa reste encore indéterminée, nous pouvons penser que tous ne représentent pas des parasites invasifs et destructeurs pour la biodiversité. Certains d'entre eux pourraient être des éléments clés parfaitement intégrés aux réseaux trophiques et assurant le maintien de la diversité comme cela pourrait être le cas selon l'hypothèse « Killing The Winner ». Pour comprendre pleinement la dynamique et l'incidence des parasites appartenant aux Perkinsozoa, différents niveaux d'étude (individuelle,

populationnelle, communautaire, écosystémique et spatio-temporelle) doivent être combinés. Pour cela, aucune des approches *in situ* et *in vitro* ne doit être dévalorisée car certaines sont essentielles à la résolution de certains mécanismes et processus se déroulant à des échelles spécifiques (Figure 2). Dans le cas des Perkinsozoa, une coopération entre les domaines de la pathologie, de la biologie moléculaire et de la modélisation permettrait de résoudre le rôle de ces organismes dans les écosystèmes, qu'ils soient libres ou symbiontes (interaction parasitique, mutualiste ou symbiotique).

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Annexe 1

Pathology and Case Definition of Severe Perkinsea Infection of Frogs

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Abstract

Severe Perkinsea infection (SPI) is an emerging disease of frogs responsible for mass mortalities of tadpoles across the United States. It is caused by protozoa belonging to the phylum Perkinsozoa that form a distinct group referred to as the Pathogenic Perkinsea Clade of frogs. In this work, we provide detailed description of gross and histologic lesions from 178 naturally infected tadpoles, including 10 species from 22 mortality events and 6 amphibian health monitoring studies from diverse geographic areas. On external examination, we observed abdominal distension (10, 5.6%), cutaneous erythema and petechia (3, 1.7%), subcutaneous edema (3, 1.7%), and areas of white skin discoloration (3, 1.7%). On macroscopic examination of internal organs, we found hepatomegaly (68, 38.2%), splenomegaly (51, 28.7%), nephromegaly (47, 26.4%), ascites (15, 8.4%), segmental irregular thickening and white discoloration of the intestine (8, 4.5%), pancreatomegaly (4, 2.2%), and pancreatic petechia (1, 0.6%). Histologically, over 60% of the liver (148/165, 89.7%), kidney (113/147, 76.9%), spleen (96/97, 99%), and pancreas (46/68, 67.6%) were invaded by myriad intracellular and extracellular Perkinsea hypnospore-like and trophozoite-like organisms. Numerous other tissues were affected to a lesser extent. Mild histiocytic inflammation with fewer lymphocytes or eosinophils was commonly observed in areas of infection that were not obscured by lympho-granulocytic hematopoietic tissue. In light of these observations, we suggest a logical pathogenesis sequence. Finally, we propose a “case definition” for SPI to promote standardized communication of results and prevent misdiagnosis with epidemiological and pathologically overlapping diseases such as ranaviriosis.

Keywords

amphibian, case definition, emerging infectious disease, histopathology, pathogenesis, pathogenic perkinsea clade, protists, ranavirus, tadpoles

Nearly one-third of all known amphibian species are threatened and over 400 are critically endangered.³⁴ Since the 1970s, over 200 frog species have gone extinct, and hundreds more may face extinction in the coming decades.¹ Along with habitat degradation, emerging infectious diseases have an important role in this worldwide decline of frog populations.¹

In North America, chytridiomycosis and ranaviriosis are responsible for mass mortalities of anurans.^{11,16,25,36} Whereas chytridiomycosis mostly targets postmetamorphic frogs,^{11,36} ranaviriosis principally affects premetamorphic and metamorphic stages (hereafter referred as tadpoles).^{16,25}

In the last 2 decades, another infectious disease of tadpoles, severe Perkinsea infection (SPI), has been recognized as causing mass mortalities across the United States from boreal to subtropical areas.^{6,9,20,22} The detection of SPI mortality in Alaska and other northern states suggests that this disease is likely distributed throughout much of the Nearctic region.²⁰ Like ranaviriosis,³⁵ SPI can threaten susceptible frog species such as the critically endangered dusky gopher frog (*Rana sevosa*), by drastically reducing recruitment.^{17,20}

Severe Perkinsea infection is caused by protozoa belonging to the phylum Perkinsozoa (superphylum Alveolata). A recent

phylogenetic analysis of isolates from SPI-affected tadpoles across the United States showed that all examined sequences of the small subunit of the ribosomal RNA-encoding gene clustered into a highly supported clade tentatively named Pathogenic Perkinsea Clade of frogs (PPC).²⁰ Although little is known about the etiologic agent of SPI, other Perkinsea species within the genus *Perkinsus* (eg, *P. marinus*, *P. olseni*), responsible for mass mortalities of mollusks and secondary

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economic losses, have been well studied.¹² These *Perkinsus* spp. have a direct life cycle, with a free-living aquatic zoospore (infective) stage.² After infection, the zoospore becomes a trophozoite that proliferates through host tissues by vegetative reproduction. In moribund or dead hosts, the trophozoite enlarges and develops a thick wall, thus becoming a hypnospore (resting stage) that endures outside the host in sediments.^{27,37} Finally, zoosporulation occurs when the hypnospore is resuspended into the water column or ingested by a new host.³

The relatively recent acknowledged threat that emerging infectious diseases represent for frog populations has led to increased monitoring of mortality events.⁸ Yet, incomplete postmortem investigations may lead to misdiagnosis given the epidemiological and pathological similarities of some amphibian diseases and the existence of subclinical carriers.^{10,16,20,28}

Establishing a case definition is one route to standardize diagnostic results, promote consistent reporting of emerging diseases, and aid in differentiating diseases with overlapping diagnostic criteria. Although standard in human medicine, case definitions are uncommon for wildlife diseases. A recent case definition of the emerging amphibian chytridiomycosis of caudates caused by *B. salamandrivorans* was an important step in organizing diagnostic communications for a newly identified disease.³⁸ Case definitions typically include field, gross, histopathology, laboratory, and epidemiologic criteria for assigning a specific disease, condition, or presence of a pathogen, as well as confidence in a diagnosis given the current state of knowledge and evidence from available diagnostic criteria.

In previous publications, we provided a general outlook of frequent pathological changes associated with SPI.^{19,20} In this article, we expand upon that previous work to provide more detailed macroscopic and histologic pathology descriptions of a large set ($n = 178$) of wild frogs with SPI, with special attention to frequency and severity of lesions in each organ. In light of these observations, we propose a plausible pathogenesis sequence. We also discuss the importance of complete pathologic evaluation during anuran disease investigations given the epidemiological and pathological similarities among SPI and other diseases of tadpoles such as ranaviriosis. Finally, we propose a case definition of SPI based on current knowledge of the disease to standardize communications and reduce misdiagnosis.

Methods

We reviewed pathology reports and hematoxylin and eosin-stained histologic slides of 178 tadpoles from 22 SPI-associated mortality events and 6 amphibian health monitoring studies (mortality not specifically tracked or estimated) in 13 states of the United States from 1999–2017. Severe Perkinsea infection was confirmed in each of these 178 tadpoles by Isidoro-Ayza et al.^{19,20} Postmortem investigation took place at the USGS-NWHC (Madison, Wisconsin). Confirmation of SPI was based on histological observation of pathological changes in tissues with presence of Perkinsea-like organisms

in at least 1 organ, including brain, eyes, gastrointestinal tract, gills, heart, liver, lungs, kidney, pancreas, skeletal muscle, skin, spinal cord, spleen, and thyroid gland.

Anuran species and developmental stage identification was confirmed based on external morphological features.^{14,29,33}

For histopathology, samples were fixed in 10% formaldehyde for at least 48 h. Specimens with partially or completely ossified skeleton, were decalcified in formic acid-sodium citrate mixture overnight. Fixed samples were dehydrated with a graded ethanol series, embedded in paraffin and sectioned at a thickness of 4- μ m with a rotary microtome, then stained using Mayer's hematoxylin and eosin. For each carcass, the overall extent (as percentage) and distribution of the histologic lesions on hematoxylin and eosin-slides, the distribution of the Perkinsea-like organisms in different organs (including trophozoite-like and hypnospore-like organisms), and the estimated ratio of trophozoite-like to hypnospore-like structures in liver were assessed by a board-certified veterinary pathologist (MIA).

For electron microscopy, a fragment of liver and spleen from 1 SPI-affected tadpole (USGS-NWHC identification 28207-015); and kidney from another SPI-affected tadpole (28207-012) were preserved in paraformaldehyde-glutaraldehyde solution (Kamovsky's fixative), after which they were dehydrated in a graded ethanol series, infiltrated with epoxy propylene oxide, and embedded in epoxy resin. The epoxy block was sectioned with an ultra-microtome at a thickness of 1- μ m to produce semi-thin sections that were thereafter stained with uranyl acetate followed by lead citrate and examined with a transmission electron microscope equipped with a digital photomicrograph (Hemmatsu ORCA HR Camera). Electron microphotographs of liver from 2 SPI-affected tadpoles (4957-288 and 16406-009)²⁰ were reviewed for the present study.

In previous studies,^{19,20} PPC was confirmed by PCR amplification and sequencing of the small subunit of the ribosomal RNA-encoding gene in 19 of these 178 tadpoles, including at least 1 tadpole from 12 of 19 Counties with histologically confirmed SPI events (10 out of 13 states) (Supplemental Table S1). In addition, previous to fixation, kidney, chilled or frozen and thawed liver, or pooled internal organs of 44 of these 178 tadpoles from 14 SPI mortality events and 1 amphibian health monitoring study were tested for frog virus-3 as previously described,^{23,24} with only 1 tadpole being positive.^{19,20}

Fresh liver or spleen from 27 of the 178 tadpoles from 8 SPI mortality events and 2 amphibian health monitoring studies were processed for bacterial testing. Briefly, aseptically collected specimens were inoculated onto blood and MacConkey agar plates and incubated at 37C. All isolates were identified using API strips (BioMerieux, Marcy l'Etoile, France) or the OmniLog microbial identification system (Biolog Inc, Hayward, CA, USA).

All samples used for this study were from tissue archives and originated from wildlife disease investigations conducted on amphibian carcasses from 1999 to 2017. Euthanasia of frogs included in this study was approved by USGS National

Wildlife Health Center Institutional Animal Care and Use Committee (IACUC) protocol EP080707.

Results

Life Stage and Body Condition

Tadpole life stage¹⁴ was determined and reported in 166 of 178 SPI-affected tadpoles (93.3%). Thirteen (7.8%) were late hatchlings (Gosner stage 25), 135 (81.3%) were larval stages (Gosner stages 26-41) and 18 (10.8%) were metamorphs (Gosner stages 42-46). No adult frogs were diagnosed with SPI. Body condition was reported in 113 tadpoles and was good in 85 (75.2%) and poor in the remaining 28 (24.8%).²⁰

Macroscopic Lesions

On gross examination, a subset of 10 tadpoles (5.6%) presented marked abdominal distension. Skin lesions were observed in 9 tadpoles (5.1%), including multifocal, irregular redness of the skin (erythema) and pinpoint hemorrhages (petechia) (3, 1.7%) (Fig. 1); subcutaneous bilateral symmetric edema of the abdominal region, base of the tail, inguinal area and thighs (Fig. 2) (3, 1.7%); and randomly distributed 2 to 10-mm areas of white discoloration (3, 1.7%). On internal examination, 15 tadpoles (8.4%) presented moderate to abundant accumulation of serous fluid in the coelomic cavity (ascites); 68 tadpoles (38.2%) presented enlargement of the liver with rounded edges (hepatomegaly) and mottled pale discoloration (Fig. 3); 51 tadpoles (28.7%) presented enlarged spleen (splenomegaly) with mottled pale or red discoloration (Fig. 3); 47 tadpoles (26.4%) presented increased size of the kidney (nephromegaly), including pronephros and mesonephros, with pale or red discoloration (Fig. 3); the intestine of 8 carcasses (4.5%) presented segmental to coalescing areas of irregular thickening and white discoloration of the intestinal wall (Fig. 4); and 5 tadpoles (2.8%) presented lesions in the pancreas were noted, including moderate enlargement of the organ (pancreatomegaly) and diffuse orange discoloration (Fig. 3) (4, 2.2%), or multifocal petechial hemorrhages (1, 0.6%).

Histologic Lesions

Histologic lesions were associated with invasion of numerous tissues by myriads of intracellular and extracellular Perkinsea-like organisms. All the organs routinely examined presented variable invasion by these organisms and secondary tissue damage. The most extensively affected organs were liver, spleen, kidney and pancreas. The extent of the lesions in these 4 organs from the 178 tadpoles included in this study is summarized in Supplemental Table S2. In addition, 1 or more of the following tissues were invariably affected but to a smaller extent; cartilage, central nervous system, gastrointestinal tract, gills, gonads, heart, peritoneum, skeletal muscle, skin, and thyroid glands.

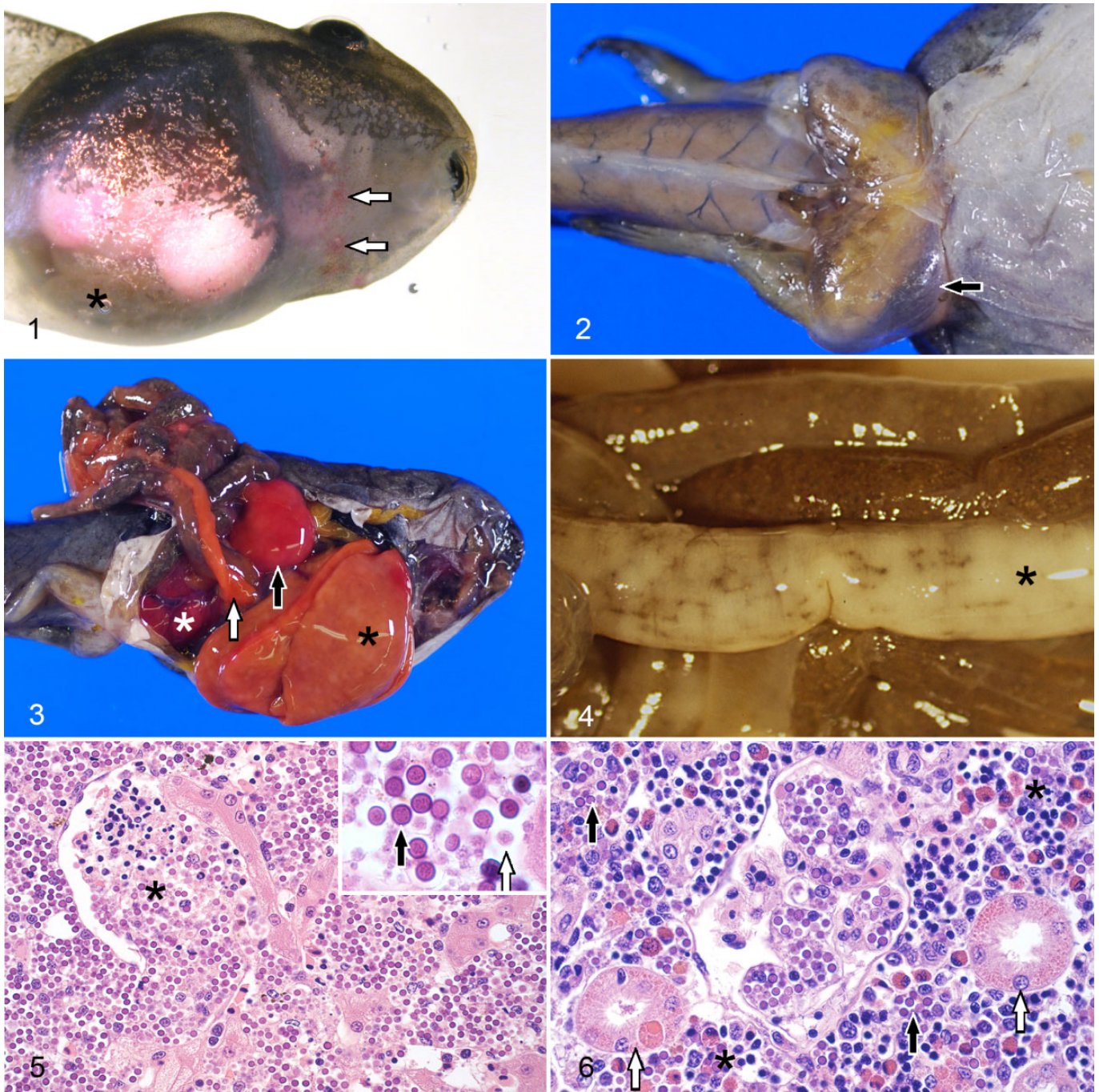
Most sections of liver examined presented random, multifocal-coalescing replacement of over 60% (148/165,

89.7%) of the hepatic parenchyma and varying disruption of Glisson's capsule by myriads of Perkinsea-like organisms (Fig. 5). Two life stages were observed: 4- μ m diameter spherical structures with thick deep basophilic wall, and granular pale basophilic cytoplasm (hynospore-like); and 2- μ m diameter, amoeboid, pale basophilic organisms (trophozoite-like) (Fig. 5, inset). The hynospore-like stage was the more abundant (>50% of the organisms in 3 random 100X fields) in 126/162 (77.8%). The Perkinsea-like organisms were embedded in variable amounts of necrotic debris, fibrin, and erythrocytes. Sparse macrophages with occasional intracytoplasmic Perkinsea-like organisms and varying numbers of lymphocytes and eosinophils were observed in the affected tissues. Granulopoietic tissue with predominance of the eosinophil series, characterized by a nonlobed to bilobed eccentric nucleus and numerous large round bright eosinophilic cytoplasmic granules (Supplemental Fig. S1), was commonly observed in portal spaces. In less severely affected areas, sinusoids were multifocally expanded and variably obliterated by Perkinsea-like organisms. The hepatic cords were dissociated and in many instances hepatocytes contained up to 4 Perkinsea-like organisms in their cytoplasm. Endothelial cells in sinusoids and small blood vessels were occasionally missing, and clusters of the same organisms, fibrin, necrotic debris and erythrocytes were attached to the exposed subendothelial surface (thrombi) (Fig. 5). In some instances, medium to large areas of the organ showed coagulative necrosis and occasional presence of nearby thrombi. Infected and uninfected hepatocytes presented hydropic degeneration or atrophy. Medium- and large-size blood vessels and bile ducts showed variable cholestasis.

In the kidney (pronephros and mesonephros), Perkinsea-like organisms infiltrated the intravascular and perivascular interstitial space and the glomerular tufts, in most instances replacing over 60% (113/147, 76.9%) of the renal tubules and glomeruli (Fig. 6). Remaining tubules showed variable attenuation, hydropic degeneration or intracellular eosinophilic, hyaline, droplets (protein reabsorption) and necrosis of the epithelial cells, and in many cases, intracytoplasmic invasion by the infectious agent. Multifocal, interstitial lympho-granulopoietic tissue with dominant eosinophil series was prominent in most instances, and inflammatory infiltrates were generally not assessable.

In the spleen, mostly affecting the red pulp with extension into the white pulp, Perkinsea-like organisms infiltrated the intravascular and perivascular spaces often replacing 60% of the organ (96/97, 99%), and embedded in variable amounts of necrotic debris. The same scattered inflammatory infiltrates described in the liver were also observed in this organ.

In the pancreas, intravascular and extravascular Perkinsea-like organisms expanded the interstitium and replaced the exocrine epithelium of over 60% of the organ in most tadpoles (46/68, 67.6%). Perkinsea-like organisms were occasionally observed in the cytoplasm of exocrine pancreatic epithelial cells which showed variable degrees of hydropic degeneration. Scattered histiocytic and lympho-eosinophilic inflammatory



Figures 1–6. Severe *Perkinsea* infection (frog virus-3 negative), frog. **Figure 1.** Wood frog (*Rana sylvatica*) larva (Gosner stage 30), accession 19456-001 (Kenai Peninsula, Alaska, June 2005 outbreak). There are multiple petechial hemorrhages in the skin of the gular area (arrows). There is presence of subcutaneous edema in the ventral area of the body (asterisk). **Figure 2.** Green frog (*R. clamitans*) larva (Gosner 41), accession 28207-007 (Vilas, Wisconsin, July 2017 outbreak). There is bilateral subcutaneous edema in the inguinal region of both rear limbs (arrow). **Figure 3.** Green frog, accession 28207-006 (Vilas, Wisconsin July 2017 outbreak). There is marked enlargement of the liver with rounded edges (hepatomegaly) and mottled pale discoloration (black asterisk); increased size of the spleen (splenomegaly) and red discoloration (black arrow); mesonephros appears swollen (nephromegaly) and presents diffuse red discoloration (white asterisk). Pancreas is prominent and mottled pale orange (white arrow). **Figure 4.** Intestine, dusky gopher frog (*R. sevosa*), accession 18587-008 (Harrison, Mississippi, March 2003 outbreak). There are multifocal-coalescing areas of irregular thickening and white discoloration of the intestinal wall (asterisk). **Figure 5.** Liver, green frog tadpole, accession 28207-15 (Vilas, Wisconsin, July 2017 outbreak). Over 60% of the liver is replaced by necrotic debris and *Perkinsea*-like organisms. One sinusoid is expanded and partially obliterated by a fibrin thrombus with embedded *Perkinsea*-like organisms (asterisk). Isolated or small groups of remaining hepatocytes are atrophied or undergoing hydropic degeneration. Hematoxylin and eosin (HE). *Inset.* There are 4- μ m-diameter spherical structures with a thick, deep basophilic wall and granular pale basophilic cytoplasm

infiltrates similar to those described in the liver and spleen were also observed in the affected portions of pancreas.

The branchial and oral lamina propria and the gastrointestinal lamina propria and submucosa were expanded by intravascular and perivascular Perkinsea-like organisms and mild to moderate perivascular edema with variable attenuation, degeneration and necrosis of the lining and glandular epithelium (Figs. 7 and 8). Frequently, Perkinsea hypnospore-like organisms were also present in the lumen of the buccal cavity and gastrointestinal tract. Mild histiocytic inflammation with fewer lymphocytes or eosinophils was occasionally observed in affected areas. In some instances, granulopoietic tissue with predominant eosinophil series was observed in the mesenteric vessels.

Intravascular and perivascular, small to extensive infiltrates of Perkinsea-like organisms were observed in the superficial dermis. The epidermis was rarely invaded by Perkinsea-like organisms and presented variable degeneration and necrosis of the affected keratinocytes (Fig. 9). In some tadpoles, the endothelium lining subcutaneous lymphatics was disrupted and replaced by clusters of Perkinsea-like organisms and necrotic debris that were partially obliterating the lumen (Fig. 10). With the same angiocentric pattern, infiltrates of this parasite were also observed in the endomysium and perimysium of the skeletal muscle (Fig. 11), endocardium and myocardium, peritoneum, leptomeninges and choroid plexus of the brain, and very rarely in the capillaries of the neuroparenchyma and vascular layer of the retina (choroid coat). In all these tissues, presence of the organism was associated with variable perivascular to diffuse edema, endothelial activation and loss, as well as degeneration and necrosis of the myocytes, myocardiocytes, mesothelial cells, or choroid cells. The same type of inflammatory infiltrates observed in the gastrointestinal tract and gills was occasionally observed in dermis, skeletal muscle, and peritoneum.

On transmission electron microscopy, hypnospore-like and trophozoite-like organisms ultrastructurally similar to those previously described²⁰ were seen free in extracellular and virtual necrotic spaces, and intracellularly in hepatocytes, renal epithelial cells, endothelial cells, and within the cytoplasm of macrophages, endothelial cells and epithelial cells (hepatocytes and tubular epithelial cells) (Fig. 12).

Coinfections and Concomitant Pathology

Fifty-nine SPI-affected tadpoles (33.1%) presented concomitant infections. In 37 tadpoles (20.8%), there were normal intestinal burdens of intestinal metazoa including pinworm morphologically compatible with *Gyrinicola* sp. (33), intestinal cestodes (1) or both (3). Metacercariae encysted in different

tissues were observed in 24 tadpoles (13.5%). In 21 tadpoles (11.8%), metacercariae in the mesonephroi were identified as *Echinostoma* sp., and in 6 tadpoles (3.37%) metacercariae in the dermis were identified as *Riberoia ondatrae*. Twenty-three (12.9%) presented ectoparasitic infestations with *Leptotheca* sp. (19), *Trichodina* sp. (3), or *Epistilis* sp. (1). *Ichthyophonus* sp. cysts with no or mild associated granulomatous myositis were observed in 8 tadpoles (4.5%). Seven tadpoles presented mild focal hyperkeratosis of the oral disc with intralesional chytrid-like thalli (zoosporangia) (3.9%). Necrotizing dermatitis and stomatitis caused by *Saprolegnia* spp. was diagnosed in 4 tadpoles (2.2%). We previously²⁰ isolated and amplified frog virus-3 DNA in 1 of 44 tadpoles that were tested for this pathogen (2.3%). *Myxidium* sp. infections were detected in the liver of 2 tadpoles (1.1%). Routine culture of liver (25) or spleen (2) from 27 tadpoles resulted in no bacterial growth indicative of ante-mortem bacterial infection. Deformities of the hind limbs including absence (amelia) or underdevelopment (ectromelia), or extra number of limbs (polymelia), were observed in 3 individuals (1.7%).

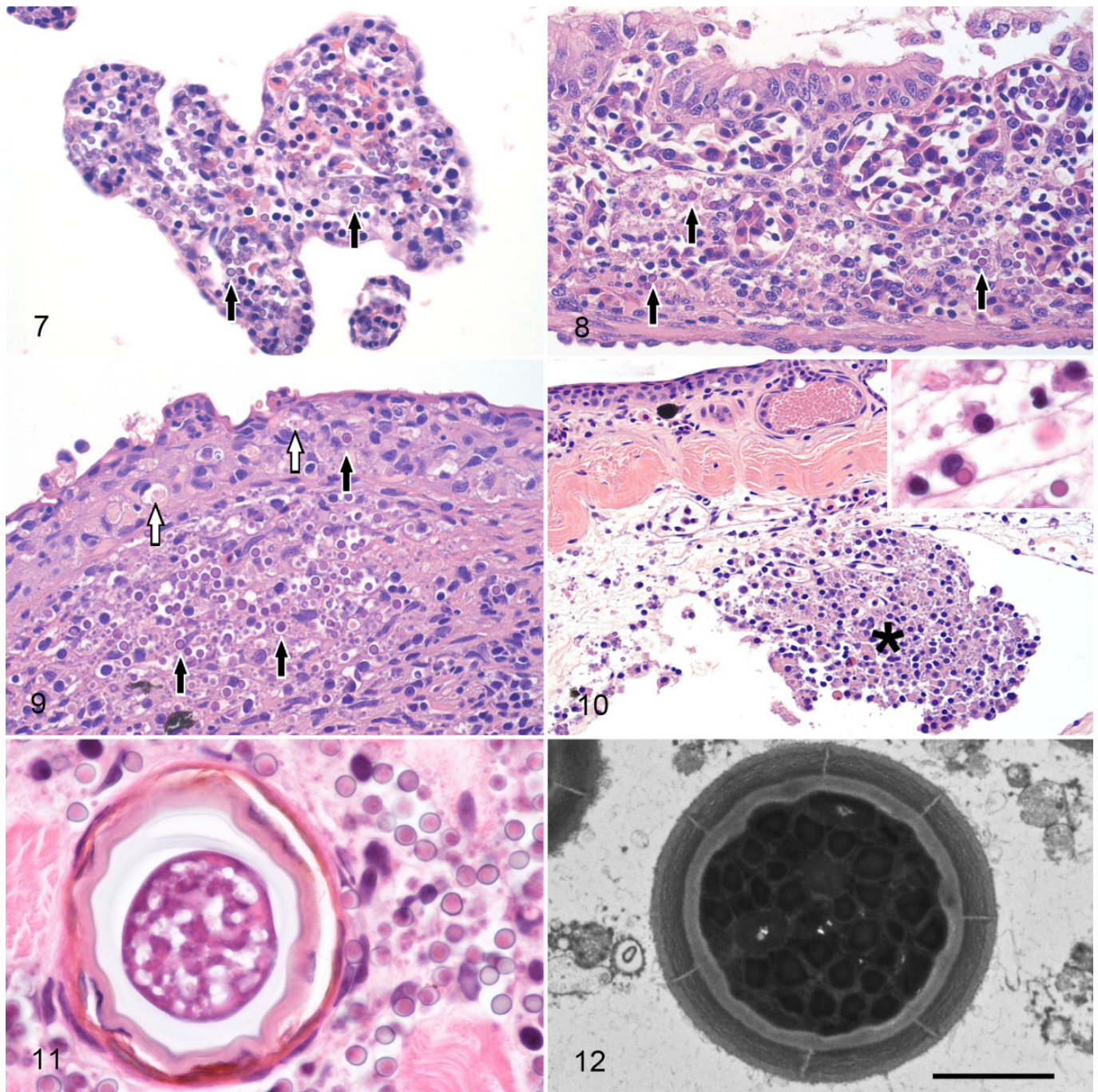
Discussion

Since first detected in 1999, SPI events have been increasingly identified as the cause of mass mortalities of tadpoles across the United States^{9,19,20,22} and represent, after ranavirosis, the second most frequently diagnosed life threatening infectious disease of tadpoles in the United States.²⁰ Like ranavirosis,³⁵ SPI can cause over 95% mortality in tadpole populations and has the potential to drive fragmented frog populations to extinction by substantially reducing recruitment.^{17,20}

SPI Pathology

Most SPI-affected tadpoles presented with well-developed fat bodies in the coelomic cavity (good body condition), which suggests that the course of SPI is rapid and does not lead to prolonged anorexia or negative energetic imbalance.²⁰ Eighty SPI-affected tadpoles that were examined lacked gross abnormalities (44.9%). When present, the most common SPI associated gross lesions were hepatomegaly, splenomegaly and nephromegaly. Rarely pancreatomegaly was noticed. Organomegaly corresponded histologically with extensive necrosis and replacement of preexistent tissues by myriads of Perkinsea-like organisms. Tissue necrosis may be caused by the invasion and presumed replication of Perkinsea-like organisms in epithelial cells, including hepatocytes, tubular epithelial cells, pancreatic exocrine epithelium, keratinocytes, enterocytes, branchial epithelium or thyroid epithelium. In some instances, Perkinsea-like organisms were observed in the

Figures 1–6. (Continued). (hypnospore-like, black arrow) and 2- μ m-diameter, amoeboid, pale basophilic organisms (trophozoite-like, white arrow). HE. **Figure 6.** Severe Perkinsea infection, kidney, green frog tadpole, accession 28207-15 (Vilas, Wisconsin, July 2017 outbreak). There are Perkinsea-like organisms infiltrating a glomerular tuft, renal interstitium and replacing the renal tubules (black arrows). The remaining tubules show variable attenuation or intracellular eosinophilic, hyaline droplets suggestive of protein reabsorption (white arrows). Multifocal, interstitial lympho-granulopoietic tissue is prominent (asterisks). HE.



Figures 7–12. Severe *Perkinsella* infection. **Figure 7.** Gills, green frog tadpole, accession 28207-15 (Vilas, Wisconsin, July 2017 outbreak). The branchial interstitium is expanded by *Perkinsella*-like organisms (arrows). The lining epithelium is attenuated or absent. Hematoxylin and eosin (HE). **Figure 8.** Intestine, green frog tadpole, accession 28207-15 (Vilas, Wisconsin, July 2017 outbreak). The lamina propria is invaded and expanded by *Perkinsella*-like organisms (arrows). Multifocally, enterocytes are detached from the basement membrane. Blood vessels are engorged (congestion), and plump mesothelial cells line the gastric serosa (mesothelial cell activation). Notice that the absence of villi and crypts is characteristic of tadpole stages since these features develop by the end of metamorphosis. HE. **Figure 9.** Skin, green frog tadpole, accession 28207-15 (Vilas, Wisconsin, July 2017 outbreak). *Perkinsella*-like organisms are present in the dermis, and to a lesser extent in the epidermis (black arrows). In the epidermis, there is hydropic degeneration and necrosis of multiple keratinocytes (white arrows). In the dermis, surrounding the area invaded by the protozoan, there are lympho-histiocytic inflammatory infiltrates. HE. **Figure 10.** Skin and subcutis, green frog tadpole, accession 28207-15 (Vilas, Wisconsin, July 2017 outbreak). The subcutis is edematous and is invaded by histiocytes and *Perkinsella*-like organisms (at the left). In the center, a lymphatic is partially obliterated by a cluster of *Perkinsella*-like organisms, macrophages and necrotic debris attached to the subendothelium (asterisk). HE. *Inset.* Higher magnification showing a subcutaneous capillary. There is an intraluminal monocyte containing a *Perkinsella* hypospore-like organism in its cytoplasm and a lymphocyte undergoing margination.

cytoplasm of endothelial cells. This finding, along with the observation of endothelial loss and thrombosis suggests that vascular damage induced by the protozoa might partially contribute to hypoxic degeneration and necrosis. Eosinophilic granulopoiesis was frequently noticed in kidney, liver, and mesenteric tissues, while lymphopoiesis was mostly observed in kidney. In addition, SPI-affected tadpoles presented with increased numbers of circulating eosinophils (eosinophilia). In immature stages of some frog species, elevated production and deployment of eosinophils might be a physiological change that responds to the high demand of eosinophils necessary for tissue remodeling.¹³ The exuberant extramedullary hematopoiesis and eosinophilia observed in many of the examined SPI-affected tadpoles probably concealed underlying mild inflammatory infiltrates. For infected tissues in which the cellular inflammatory response was not obscured by hematopoietic tissue, such as skin, specific inflammatory infiltrates were minimal or absent. When present, inflammatory cells were scattered histiocytes with occasional intracytoplasmic Perkinsea-like organisms, fewer lymphocytes and eosinophils (Inset Fig. 10). Contrary to adult frogs which appear to confine the Perkinsea-like organisms in granulomas,²¹ widespread presentation of SPI in tadpoles suggests inability of the tadpole immune system to control the infection. The high susceptibility of tadpoles to SPI might be partially explained by incomplete development of their adaptive immune system or down-regulation of immune responses during metamorphosis.³⁰ Moreover, environmental and ecological factors such as water temperature might impair immune response of tadpoles to PPC.⁴ Nonetheless, mild but efficient granulomatous tissue confinement of metazoan and other intracellular protozoa such as *Ichthyophonus* sp. observed in SPI-affected tadpoles suggests that tadpoles are capable of mounting an immune response to pathogens and that PPC might use alternative mechanisms to evade the host immune response (Fig. 11).

Two other gross findings observed in SPI were ascites and subcutaneous edema. In SPI, edemas may be partially explained by a decrease of the oncotic pressure secondary to hypoalbuminemia following extensive acute hepatic necrosis.⁷ Likewise, acute renal failure secondary to glomerular and tubular damage probably contributes to the formation of edemas by eliciting protein loss,⁷ which was observed in this study as eosinophilic protein droplets in the renal tubular epithelium of SPI-affected tadpoles. In addition, edemas might be caused in part by massive sinusoidal and vascular obliteration by Perkinsea-like organisms, which significantly increases sinusoidal and systemic hydrostatic

pressure.⁷ Endothelial damage and increased vascular permeability may also partially contribute to the formation of edemas and occasionally observed petechial hemorrhages in skin and pancreas.

The predominant protozoal stage identified in natural SPI was the hypnospore-like stage. However, in 7/15 (46.7%) of the SPI-affected tadpoles that presented with less than 60% of liver affected (earlier stages), trophozoite-like organisms were the dominant stage. Unfortunately, most tadpoles collected in passive surveillance studies are in terminal stages of the disease (moribund or dead), which hampers assessment of the disease progression. In the better studied *Perkinsus* spp. of mollusks, hypnospore life stages are only observed in moribund or dead specimens.^{3,27} The transformation from trophozoite to hypnospore is triggered by chemical changes in the decaying tissues such as decreased oxygen tension and pH.⁵ Likewise, in SPI, extensive necrosis and multiorgan failure might elicit massive and rapid transformation of the trophozoite-like stage into hypnospore-like stage. Thereafter, the hypnospore-like stage presumably persists under variable conditions in the decaying tissues, in the environment (water or sediments) or in the digestive tract and feces of a paratenic host. To confirm this hypothesis, controlled infections for serial durations followed by light and electron microscopy need to be conducted.

Despite limitations associated with the opportunistic nature of our samples, a logical proposed course of SPI involves infection of the tadpole by zoospores through the oral mucosa and gills, gastrointestinal tract or percutaneous; local replication of trophozoite-like organisms and systemic active or passive migration within leukocytes (most likely monocytes) through blood vessels and lymphatics (Inset Fig. 10) as reported for other Perkinsea such as *Perkinsus marinus*, which infects and replicates within the hemocytes of infected oysters;³² and secondary replication in epithelial and endothelial cells of multiple organs causing degeneration and necrosis. Mild histiocytic inflammation and phagocytosis of organisms by local macrophages is probably evaded by these Perkinsea organisms as described for other well-known Perkinsozoa.³² Extensive disruption of liver and kidney cause hepatic and renal failure, occasionally leading to generalized edema. Edema might also be aggravated by the endothelial damage of blood vessels and lymphatics. Severe pancreatic necrosis likely contributes to death by release of activated proteolytic enzymes and proinflammatory cytokines into the blood stream causing a systemic inflammatory response syndrome and shock.¹⁸ The moribund tadpoles are easily predated or scavenged after death by other tadpoles or paratenic hosts, and hypnospore-like organisms are eventually

Figures 7–12. (Continued). In the perivascular space there are 2 macrophages with intracytoplasmic organisms compatible with Perkinsea-like trophozoites. HE. **Figure 11.** Skeletal muscle of the tail, American bullfrog tadpole (Gosner 36), accession 16407-9 (Carroll, New Hampshire, September 1999 outbreak). Perkinsea-like organisms are infiltrating the endomysium and replacing the myocytes with no associated inflammation. In the center is a 70 μm -diameter parasitic cyst morphologically compatible with *Ichthyophonus* sp. The cyst has a double wall and contains basophilic globular material compatible with endospores. HE. **Figure 12.** Spleen, green frog tadpole, accession 28207-15 (Vilas, Wisconsin, July 2017 outbreak). Perkinsea hypnospore-like stage with thick trilaminar cell walls with 7 electron-dense plates separated by transmural channels, and numerous electron-dense cytoplasmic lipid vacuoles. Bar = 2 μm . Transmission electron microscopy.

Table 1. Comparison of the Epidemiology and Pathology of Natural Severe Perkinsea infection (SPI) and Ranavirosis in Tadpoles in North America.

	SPI	Both	Ranavirosis
Species reported in wild frog mortalities	Ranidae (3): Dusky gopher frog (<i>Rana sevosa</i>), gopher frog (<i>R. capito</i>), river frog (<i>R. heckscheri</i>); Hylidae (1): Florida cricket frog (<i>Acris gryllus dorsalis</i>) ²⁰	Hylidae (1): Spring peeper (<i>Pseudacris crucifer</i>); Ranidae (6): American bullfrog (<i>R. catesbeiana</i>), green frog (<i>R. clamitans</i>), mink frog (<i>R. septentrionalis</i>), northern leopard frog (<i>R. pipiens</i>), southern leopard frog (<i>R. sphenoccephala</i>), wood frog (<i>R. sylvatica</i>) ^{9,19,20,22,25}	Bufonidae (1): Boreal toad (<i>Bufo boreas boreas</i>); Hylidae (8): American green tree frog (<i>H. cinerea</i>), Cope's gray tree frog (<i>H. chrysoscelis</i>), Pacific tree frog (<i>P. regilla</i>), pickerel frog (<i>R. palustris</i>), Plains leopard frog (<i>R. blairi</i>), sierran tree frog (<i>P. sierra</i>), southern chorus frog (<i>P. nigrita</i>), spotted chorus frog (<i>P. clarkii</i>); Ranidae (6): California red-legged frog (<i>R. draytonii</i>), Columbia spotted frog (<i>R. luteiventris</i>), mountain yellow-legged frog (<i>R. muscosa</i>), northern red-legged frog (<i>R. aurora</i>); Scaphiopodidae (1): eastern spadefoot toad (<i>Scaphiopus holbrookii</i>) ²⁵
Developmental stage ¹⁴	Mostly larval stages (Gosner stages 26-41)	n/a	Ranids: Mostly late metamorphs (Gosner stages 44-46) ¹⁷
Mortality	n/a	Sudden and massive (100 s, 1000s) deaths across multiple species. Deaths may continue for weeks or months. ^{16,19,20,24,25}	n/a
Clinical signs	n/a	Mortality with no clinical signs, erratic swimming, buoyancy problems, or lethargy ^{19,20,24,25}	n/a
Gross findings	Pancreatomegaly, white discoloration and thickening of the intestinal wall	Good body condition and normal gastrointestinal content (rarely poor and scant gastrointestinal content), ascites, subcutaneous edema, cutaneous erythema (redness), petechia (pinpoint hemorrhages), irregular patches of skin white discoloration, hepatomegaly, splenomegaly, nephromegaly ^{19,20,24,25}	Ecchymoses (hemorrhagic blotches), hemorrhages in internal organs (especially kidney, liver, spleen, intestine and oral cavity), discrete pale foci scattered through organs ²⁴
Histologic findings	Perkinsea hypospore-like and trophozoite-like organisms ²⁰	Necrosis multiorgan ^{16,19,20,24,25}	Basophilic and occasionally eosinophilic intracytoplasmic inclusion bodies, necrosis of hematopoietic tissue ²⁵

Abbreviations: SPI, severe Perkinsea infections; n/a, not applicable.

released into the environment where they zoosporulate under appropriate conditions. Tadpoles that survive initial infection and become adults may successfully contain the parasite in localized granulomas.²¹

SPI Case Definition

To have an accurate knowledge of the progression and real impact of emerging diseases such as SPI on frog populations, we must use consistent surveillance protocols and methods. Underdiagnosis of diseases of tadpoles in sites with low herpetological or recreational activity is expected given the rapidity with which predators and scavengers remove sick or dead

tadpoles from the environment.^{15,20} However, misdiagnosis can represent another major data gap if mortality investigations are based solely on epidemiological information, gross pathology, and molecular tests. Mortality investigations that lack complete pathological examination may incorrectly connect the presence of a pathogenic agent in an individual (infection) with a deleterious health effect in this individual (disease).^{10,31}

In the particular case of SPI, its epidemiology, clinical presentation and gross pathology overlaps in many aspects with those observed in ranavirosis of anurans in North America (Table 1). Moribund tadpoles from SPI and ranavirosis outbreaks show similar and often unspecific clinical signs suggestive of systemic disease.²⁴ In addition, external lesions or

Table 2. Case Definition of Severe Perkinsia Infection Of Frogs (SPI).^{6,9,17,19,20,22}**I. Etiology, individual, place, and time:****Etiologic agent(s):** Perkinsia protozoa within the Pathogenic Perkinsia Clade of frogs (PPC)**Individual:** Immature frogs (hatchlings to late metamorphs). Mostly reported in frogs of the family Ranidae (true frogs) in addition to the Florida cricket frog (*Acris gryllus dorsalis*) and the spring peeper (*Pseudacris crucifer*) (family Hylidae).**Place^a:** To date, SPI has been diagnosed in frogs from 13 States of the USA from boreal to subtropical areas. Most SPI mortalities have been detected bordering the Atlantic Ocean and Gulf of Mexico. Severe Perkinsia Infection has also been detected on the Pacific coast (Alaska and Oregon) and in the Midwest United States (Minnesota and Wisconsin). The detection of SPI mortalities in Alaska and other northern States suggests that this disease is likely distributed throughout much of the Nearctic region. Sudden mass mortalities of hundreds to thousands of tadpoles may occur and continue for weeks or months. Outbreaks often exhibit high mortality rates and local recurrence.**Time^b:** In temperate regions, events have been mostly diagnosed between June and September, and less frequently between October and the end of November. In regions with subtropical climate SPI has been detected all year round.**II. Diagnostic description****Field:** Diagnostically compatible illness may present with death of immature frogs with no clinical signs, erratic swimming, buoyancy problems, or lethargy.**Gross Necropsy:** Diagnostically compatible postmortem findings may include: good body condition and normal gastrointestinal content (rarely poor and scant gastrointestinal content), ascites, subcutaneous edema, cutaneous erythema, petechiae, irregular patches of skin white discoloration, hepatomegaly, splenomegaly, nephromegaly, pancreatomegaly or white discoloration and thickening of the intestinal wall.**Histopathology:** Histopathological findings may include multiorgan necrosis and replacement by myriads of extracellular and intracellular Perkinsia-like protozoa. Most severely affected organs are liver, spleen, kidney (including pro- and mesonephros), and pancreas. Numerous other tissues are usually affected to a lesser extent (eg, gastrointestinal tract, gills or skin).**Other:** Subclinical or mild infections are uncommon.**III. Laboratory criteria for diagnosis****Confirmed:** Presence of histopathologic evidence suggestive of SPI **WITH** DNA amplification by PCR and subsequent identification of PPC by sequencing of the SSU rRNA gene from 1 or more internal organs (liver, kidney, and spleen as preferred organs).**Suspected:** Presence of histopathologic evidence suggestive of SPI **WITHOUT** detection of PPC DNA from internal organs.**Present:** DNA amplification by PCR and subsequent identification of PPC by sequencing of the SSU rRNA gene from 1 or more internal organs (liver, kidney, and spleen as preferred organs) **WITHOUT** histopathologic evidence suggestive of SPI (either negative findings or specimen unsuitable for analysis).

Abbreviations: SSU rRNA, small subunit of the ribosomal RNA-encoding gene.

^aPotentially worldwide.^bPotentially all year round.

abnormalities in SPI and ranavirosis are frequently absent²⁶ or are not perceptible due to postmortem changes. When external changes are observed, in many instances they are similar, namely: abdominal distension, subcutaneous edema, cutaneous erythema and petechia, or patchy pale discoloration of the skin.²⁴ One or more of these external lesions were observed in 19 (10.7%) SPI-affected tadpoles included in this study. Finally, occurrence of subclinical infections and coinfections with either Perkinsia organisms or frog virus-3^{16,20,22,24} may increase the risk of misdiagnosing these 2 diseases if histopathology is not conducted.

Establishing a case definition is 1 approach to standardize SPI diagnostic results, promote consistent reporting and prevent misdiagnosis with either well-known or novel diseases of frogs. With the current information available on SPI, we present a disease definition summarized in Table 2. This definition summarizes background information on this novel pathogen and clinical, gross and histological presentation of the disease as well as specific criteria required for its diagnosis. Based on this case definition, confirmation of SPI as cause of death requires: 1) histological evidence of pathological changes in 1 or more organs with presence of Perkinsia-like organisms; and 2) molecular detection and identification, from at least 1 affected organ, of Perkinsia protozoa belonging to the

Pathogenic Perkinsia Clade.^{6,20} Finally, in this definition we propose additional terminology to be used when the diagnostic “gold standard” cannot be reached. However, given the early stage of the study of this disease, these criteria might change following development of more specific molecular diagnostic techniques. At the same time, variations in presentation might accompany geographical, environmental, and host expansion of this disease.

Severe Perkinsia infection is a potentially emerging threat for frogs in North America. Further consistent and active surveillance is warranted to fully assess the impact of SPI on wild populations. In this article, we provide a complete review of the pathology of natural SPI, suggest a plausible pathogenesis sequence, and propose a case definition of SPI to standardized criteria for diagnosis and communications of this disease. This information will also be useful to increase awareness about the importance of broader comprehensive postmortem disease investigations including histopathologic assessment.

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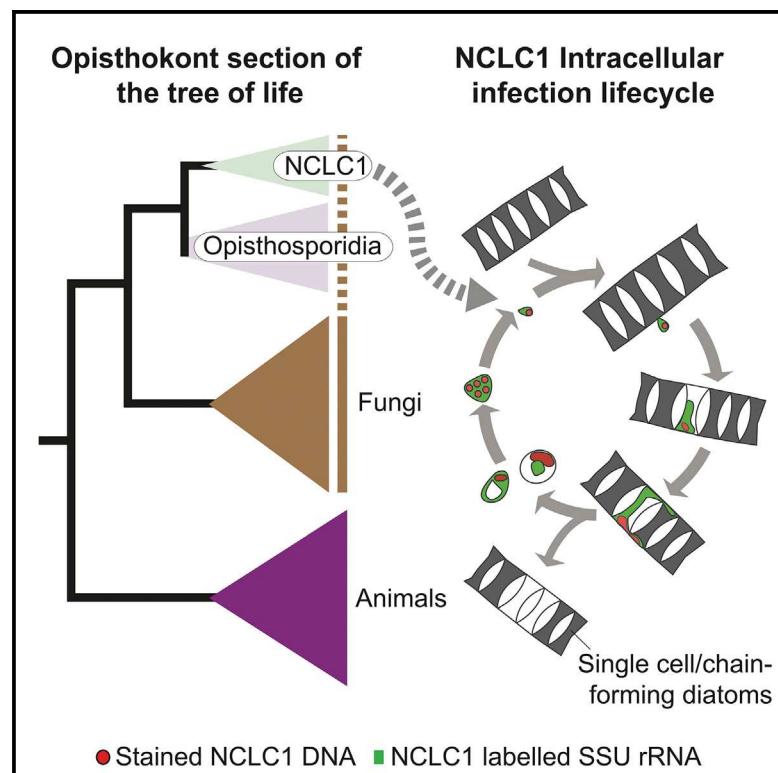
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Annexe 2

Current Biology

Intracellular Infection of Diverse Diatoms by an Evolutionary Distinct Relative of the Fungi

Graphical Abstract



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In Brief

Most microbial life remains uncultured and unstudied. Sequencing has shown a diversity of forms branching close to the Fungi. Chambouvet et al., using microscopy of labeled marine samples, show that a newly identified relative of the fungi forms intracellular infections of diatoms, potentially determining the fate of important phytoplankton blooms.

Highlights

- Environmental DNA phylogenies identify an addition to the Opisthosporidia, close to the Fungi
- This group forms intracellular infections within key marine Diatom phytoplankton
- Intracellular infection is a clade-wide lifestyle of the Opisthosporidia
- This putative parasite is found throughout the North-East Atlantic and North Sea



Intracellular Infection of Diverse Diatoms by an Evolutionary Distinct Relative of the Fungi

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SUMMARY

The Fungi are a diverse kingdom, dominating terrestrial environments and driving important ecologies. Although fungi, and the related Opisthosporidia, interact with photosynthetic organisms on land and in freshwater as parasites, symbionts, and/or saprotrophic degraders [1, 2], such interactions in the marine environment are poorly understood [3–8]. One newly identified uncultured marine lineage has been named novel chytrid-like-clade-1 (NCLC1) [4] or basal-clone-group-I [5, 6]. We use ribosomal RNA (rRNA) encoding gene phylogenies to demonstrate that NCLC1 is a distinct branch within the Opisthosporidia (Holomycota) [7]. Opisthosporidia are a diverse and largely uncultured group that form a sister branch to the Fungi or, alternatively, the deepest branch within the Fungi, depending on how the boundary to this kingdom is inferred [9]. Using culture-free lineage-specific rRNA-targeted fluorescent *in situ* hybridization (FISH) microscopy, we demonstrate that NCLC1 cells form intracellular infection of key diatom species, establishing that intracellular colonization of a eukaryotic host is a consistent lifestyle across the Opisthosporidia [8–11]. NCLC1 infection-associated loss and/or envelopment of the diatom nuclei infers a necrotrophic-pathogenic interaction. Diatoms are one of the most diverse and ecologically important phytoplankton groups, acting as dominant primary producers and driving carbon fixation and storage in many aquatic environments [12–14]. Our results provide insight into the diversity of microbial eukaryotes that interact with diatoms. We suggest that such interactions can play a key role in diatom associated ecosystem functions, such as the marine carbon pump through

necrotrophic-parasitism, facilitating the export of diatoms to the sediment [15, 16].

RESULTS AND DISCUSSION

An Addition to the Opisthokont Phylogeny

Symbiotic interactions, from parasitism through to mutualism, influence global biogeochemical processes by shaping microbial community composition and phenology [15, 17]. In marine environments, parasites have been shown to terminate algal blooms and drive phytoplankton succession [18, 19] and carbon sequestration into the deep oceans by killing algae and facilitating transit of algal carcasses down the water column [15, 16]. Diatoms can be one of the most abundant eukaryotic algae in marine environments [20], but little is known about the top-down control of these algae, particularly the role of parasitism. In freshwater and some marine ecosystems, diatoms are parasitized by zoosporic fungi, chytrids (fungi that produce spore cells with a swimming tail) [21–24]. Oomycete protist parasites have also been shown to act as a significant source of top-down control of toxic diatom bloom species [25]. However, in many marine ecosystems, the abundance and diversity of fungi, and their influence on food webs, remain poorly understood [3, 26]. The phylogenetic analyses of small subunit rRNA encoding gene (SSU rDNA) data demonstrate a diversity of sequences branching with chytrids [3, 5]. One environmental SSU rDNA group with weak phylogenetic affinities to known chytrids and which has been detected in both the sunlit water column and deep-sea sediments is the NCLC1 group [4, 5].

The opisthokonts include a huge diversity of eukaryotic forms but are composed of two major clades: (1) the animals and their protist relatives (the Holozoa) and (2) the fungi and their protist relatives (the Holomycota). Understanding the biodiversity of these groups is important for interpreting the evolutionary ancestry of these major clades (Figure 1A). The branching position of the NCLC1 group remains unresolved, with some analyses suggesting a weak association with holozoan taxa [6], while other analysis suggest that this



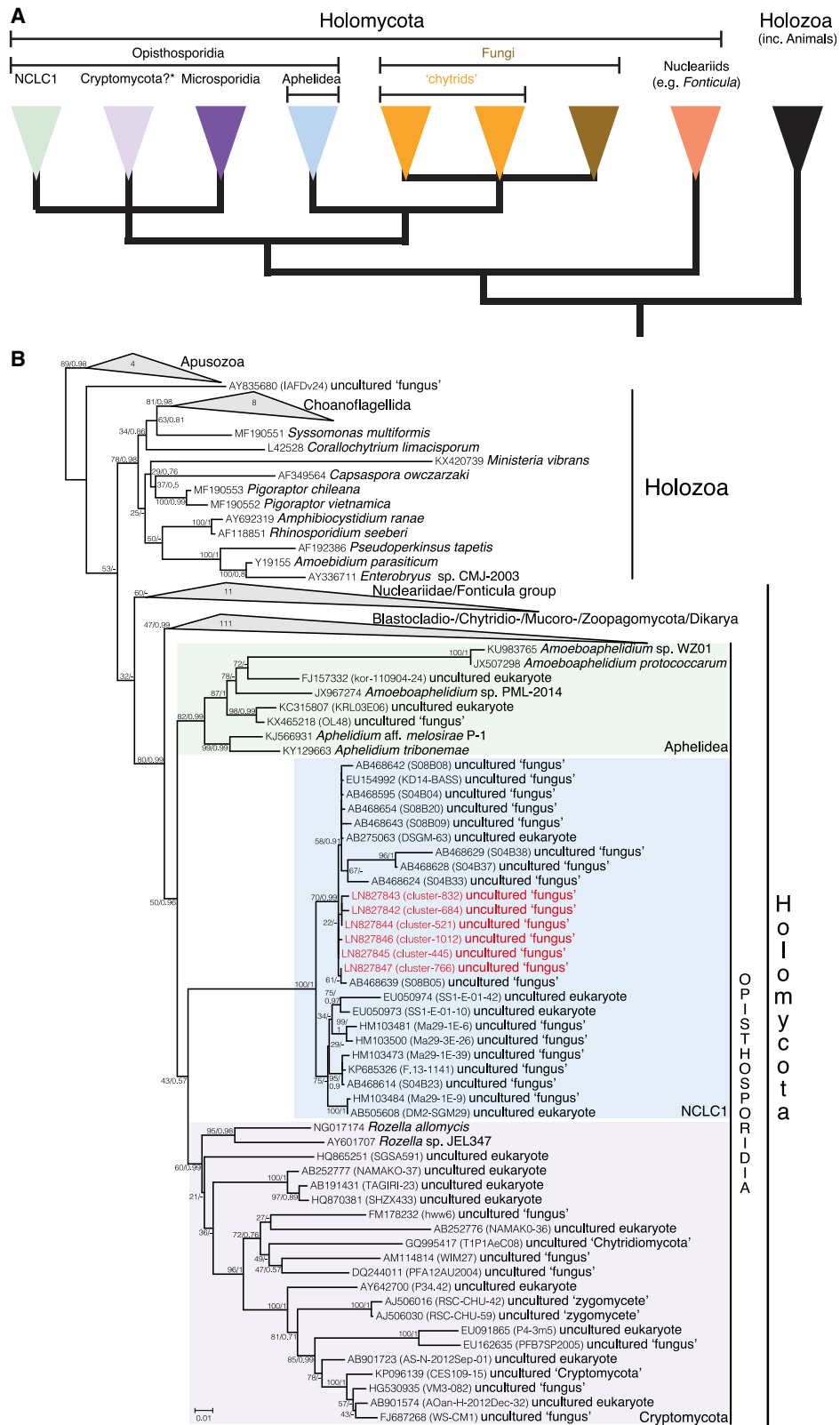


Figure 1. Phylogeny Assessing the Placement of the NCLC1 SSU rDNA Sequences Relative to the Fungi and Other Opisthokonts
 (A) Summary of the taxonomy and current best understood evolutionary relationships of the opisthokonts. Major phylogenetic relationships are based upon the phylogenetic data reported in [27].

(legend continued on next page)

group branches within the Holomycota [32] close to the Fungi [5]. Here, we investigated the phylogenetic position of the NCLC1 cluster by reconstructing maximum-likelihood (ML) and Bayesian phylogenetic trees including additional SSU rRNA gene sequences from a range of environmental DNA studies [5, 6, 33, 34]. NCLC1 sequences were found to branch sister to the Cryptomycota [35] (known variously as Rozellomycota [28], Rozellida [36], Rozellosporidia [7], or short-branch microsporidia [31]), which includes the genus *Rozella*. The NCLC1-Cryptomycota clade branches sister to the Aphelidea, with both relationships weakly supported by bootstrap analysis (Figure 1B). We recovered moderate bootstrap support (80%) for the separation of the Holomycota and the Holozoa, with the NCLC1 phylotypes clustered with the Holomycota. These results suggest that the NCLC1 represents a newly identified major group that branches with the Opisthosporidia and within the Holomycota, consistent with another analysis [31].

Fluorescent *In Situ* Hybridization Identification of NCLC1 Cells

A previous study on fungal molecular diversity in European coastal waters identified NCLC1-like sequence tags at high relative abundance compared to true fungal sequence tags at an Oslofjord (Norway) sampling site [4, 37]. NCLC1-like sequences were recovered from both large (3–20 μm and 20–1,000 μm) and small (0.6–3 μm) filtration fractions, suggesting that this group has a multifaceted life cycle, either coupled to the infection of larger cells and/or consisting of a larger, possibly multicellular life-cycle stage [4]. To explore the NCLC1 life cycle in marine environments, we used fluorescent *in situ* hybridization (FISH) microscopy to target cells from fixed filtrates sampled from the Oslofjord coastal station shown previously to harbor NCLC1 DNA/RNA diversity. Sampling was conducted to recover water from the sub-surface (1-meter depth) and deep chlorophyll maximum (DCM; 20-meter depth) fractions. From both depths, cells were recovered in two ways; water was sequentially filtered onto two different size-selective filters (0.6–3 μm and 3–20 μm) and recovered from a plankton net haul with a 1,000 μm pre-filtration sieve allowing for the recovery of cells in the range of 20–1,000 μm .

We designed three different FISH probes from the SSU V4 rRNA gene region: probe 1 (CHY-NCLC1-01), which is predicted to target the wider NCLC1 group, including OTU groups 445, 832, 521, 684, 766, and 1012 ([4]; Figure S1) and two probes (CHY-445-01 and CHY-445-02), which specifically target the NCLC1 OTU cluster 445, shown to be highly represented at Oslofjord ([4]; Figure S1). To test each probe, we used two alternative negative controls for comparison; these consisted of

either the hybridization buffer without a DNA probe or with the reverse complement of each probe. In each case, the negative controls failed to detect candidate cells.

Using the true probes, the FISH approach identified a series of candidate cells. For all sample types assayed, we observed the same cell types with the three probes in independent hybridization experiments. The FISH probes identified four variant cell forms or “cellular types” (Figures 2, 3, and S2), indicative of detection of either a heterogeneous population of microbes or a target group with multiple life-cycle phases. These cell types included an extracellular diatom association, intracellular diatom association, an un-associated, apparently free-living stage, and a multinucleated structure (also not associated with diatoms). The different FISH probe types recovered a similar percentage detection of each cell type and a similar detection profile (Figures 4A and 4B) across all filters, suggesting that the probes are independently, and consistently, detecting the same target population of cells. None of these or any other FISH-labeled cell types were identified in the negative controls.

Identification of NCLC1-Diatom Interactions

One of the four cell types observed using FISH microscopy was an irregular cellular form found inside a range of putative frustules (exoskeletons) of diatom species (Figures 2A–2F). This cell-cell association was only recovered in the 20–1,000 μm plankton net samples. Using bright-field microscopy, we discriminated the diatom’ frustules from other phytoplankton species (e.g., dinoflagellates). Using calcofluor white (CFW) staining, which preferentially labels cellulose and/or chitin cell-wall structures, e.g., on the surface of thecate dinoflagellates [18], we further excluded the possibility that NCLC1 was associating with dinoflagellates or any other cells with chitin-cellulose cell walls.

For a separate parallel water mass sample taken at the same time as the FISH samples, the diversity and abundance of the most abundant planktonic species were identified and counted using microscopy of samples fixed with Lugol’s solution [40] (Table S1; Figure S3). The combination of the taxonomic identifications obtained using diatom’ frustule analysis from the FISH microscopy and species identifications from the fixed-sample analysis allowed us to identify the taxonomy of the host groups as *Chaetoceros*, *Skeletonema*, *Pseudonitzschia*, and *Leptocylindrus* diatoms (Figures 2A–2F).

We counted the number of diatom intracellular associations observed across the two sample depths (20–1,000 μm plankton net samples taken from both the surface and DCM water samples) using the three different probes. In each case, the individual FISH hybridization experiments were replicated three times each

(B) Maximum-likelihood phylogenetic tree inferred from an SSU alignment of 200 sequences (1,221 parsimony informative sites) under the GTR+F+R6 substitution model. For collapsed groups (gray triangles), the values indicate the number of sequences present in a given group. Aphelidea, NCLC1, and Cryptomycota* (also named Rozellomycota [28], Rozellida [29], Rozellosporidia [30], and/or short-branch microsporidia [31]) are represented by green, blue, and purple rectangles, respectively. The six representative sequences recovered from the Oslo coastal station [4] are colored in red. Numbers on branches are shown in the order of bootstrap support values (percentages; computed from 100 non-parametric ML bootstrap replicates) and then posterior probabilities (inferred from two converged PhyloBayes chains). Code numbers in front of species names are NCBI - GenBank identifiers. Each collapsed branch is detailed in Table S4. The branch leading to *M. vibrans* was truncated for display purpose. SSU sequences of classically defined microsporidia are excluded from this analysis because they form excessively long branches in SSU rRNA gene trees. The phylogenetic tree is rooted on an Apusozoa outgroup; the scale bar represents the number of estimated substitutions per site. A variant reproduction of this tree is shown in Figure S1 with information about probe specificity annotated on the tree.

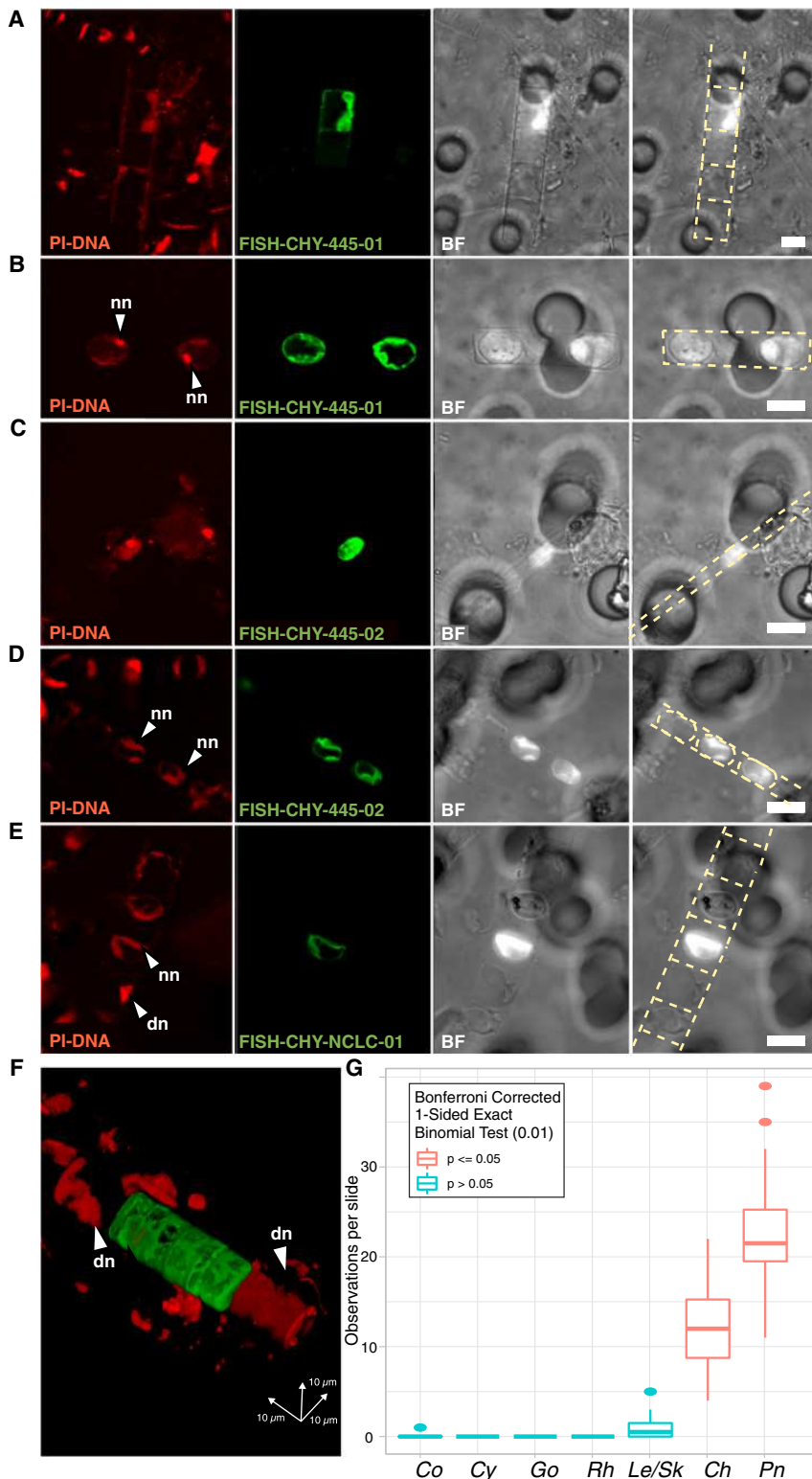


Figure 2. FISH Microscopy Evidence for NCLC1 Intracellular Associations with Diatom Phytoplankton

(A) Intracellular infection of *Chaetoceros*-like diatoms, (B) infection of *Leptocylindrus*-like diatoms, (C) infection of *Pseudonitzschia*-like diatoms, (D) infection of *Skeletonema*-like diatoms, and (E) infection of *Chaetoceros*-like diatoms. Scale bars, 10 μm . PI corresponds to nuclear DNA staining with propidium iodide; green displays cells with a positive signal for the horseradish peroxidase (HRP) FISH-labeled probes, with the specific name of the probe included on each image. BF (bright-field) corresponds to the transmitted light with differential interference phase contrast.

(F) 3D confocal reconstruction micrograph displaying an intracellular infection of a *Chaetoceros* spp. diatom by NCLC1. Figure S2 contains more details of this image, including section images showing the presence of PI staining within the parasite conglomeration but absent from the diatom carcass. We used the 3D reconstruction here and in Figure S2 to investigate the precise intracellular geography of the NCLC1 cells; these micrographs show that all DNA-containing compartments within the infected cells are surrounded by the FISH probe, suggesting that they are NCLC1 nuclei. Furthermore, these DNA structures appear more condensed compared to the nuclei of the parallel uninfected diatoms present in the filament. Based on these observations, we hypothesize that the NCLC1 has consumed the host diatom, including the nucleus; however, we cannot exclude the possibility that the NCLC1 cell(s) has encapsulated the diatom nucleus. Either interaction would suggest a parasitic association. Interestingly, these 3D reconstructions suggest that the intracellular NCLC1 cell is showing properties of amoeba-like growth; such properties have been shown for *Rozella* [38] and *Aphelids* (e.g., [7]), and some bona fide fungal chytrids also show amoeba-like crawling [39]. Some of the micrographs show internal illumination of the diatom frustule. We note that (1) we did not see this in any of the negative controls and (2) the 3D reconstruction demonstrates that some of this is associated with cellular amoeba-like extensions of the NCLC1 cell, and (3) we hypothesize that some of this signal is derived from the reflection of the strong FISH light signal off the internal glass structure of diatom frustule. Importantly, this signal is absent from uninfected diatom cells next to the infected cells on the same filament (see Figure S2). Micrographs were obtained using a Confocal Zeiss LSM780 microscope. Putative diatom nuclei are marked with an arrowhead and are labeled “dn,” and putative NCLC1 nuclei are marked with an arrowhead labeled “nn.” (G) Distribution of different observed extracellular NCLC1-diatom associations from FISH counts of the two water samples. Counts were summed per slide and probe type and then ordered by median values. Significance testing was performed per interaction under a corrected binomial test with

blue indicating an adjusted p value > 0.05 and red ≤ 0.05 . Co is abbreviated from *Cocconeis* sp.; Cy, *Cylindrotheca* sp.; Go, *Goplonema* sp.; Rh, *Rhizosolenia* sp.; Le/Sk, *Leptocylindrus/Skeletonema* sp.; Ch, *Chaetoceros* sp.; Pn, *Pseudonitzschia* sp. Box and whisker plots are shown with the outliers excluded (values more than 1.5x the inter-quartile range from the 1st or 3rd quartile) and are shown as dots. The bottom of the whisker line indicates the minimum, the start of box is the first quartile, the median line is shown, the 3rd quartile is the top of box, and maximum is the top of the whisker line. See also Figure S3 and Table S1.

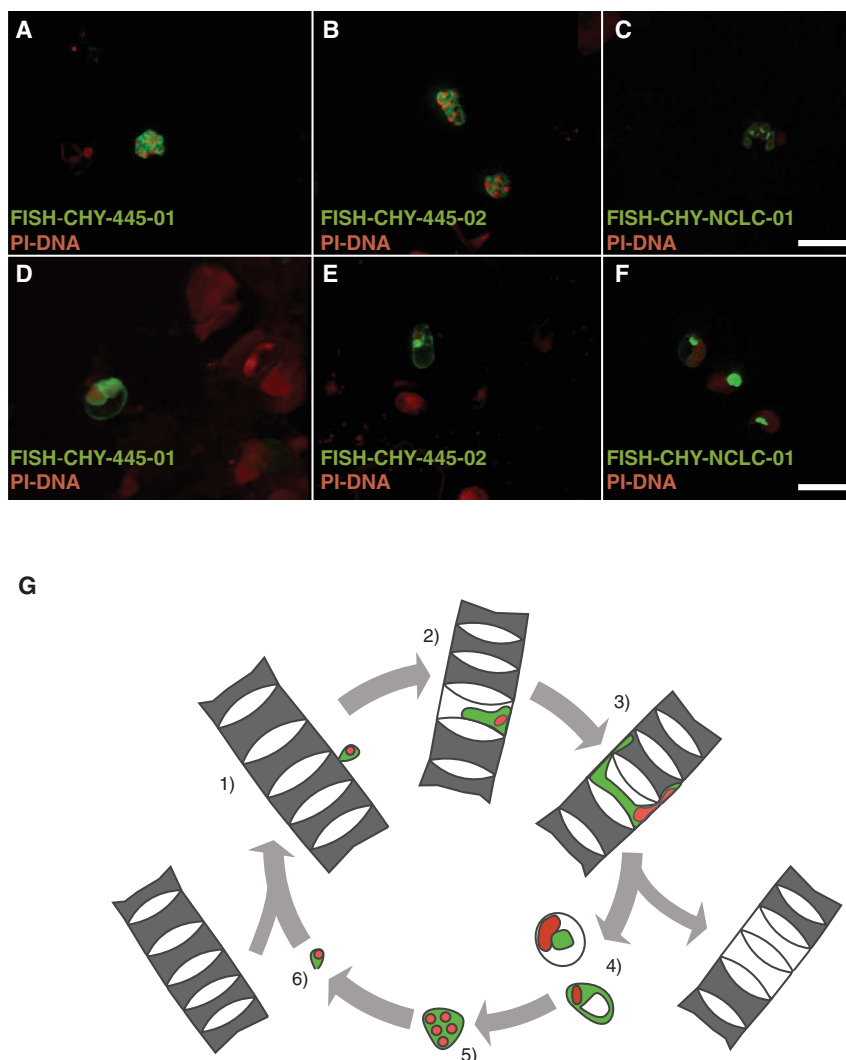


Figure 3. Merged Epifluorescence Micrographs of Additional NCLC1 Putative Life-Cycle Stages Detected and a Cartoon Showing the Inferred Life Cycle of the NCLC1 Group Identified

(A–F) FISH microscopy evidence for additional putative life-cycle phases of the NCLC1 cells identified is shown. The red and green colors correspond with the nuclear staining with propidium iodide and the FISH positive signal of the labeled probes, respectively (probes are named on the bottom corner of each image). (A–C) putative multinucleated structures and (D–F) putative spore or cyst life stage or potentially secondary host associations. The cells identified in (D), (E), and (F) are marked by sub-compartment localization of the FISH probe, suggesting either that the cyst cells have an extensive vacuole or organelle systems or that these cells represent infections of additional secondary hosts. Scale bars, 10 μ m.

(G) Cartoon illustration of the putative partial life cycle of the NCLC1 cluster 445 organisms; these are surmised from the FISH data presented and demonstrate (1) attachment to host diatoms, (2) intracellular invasion (Figures 2 and S2), (3) spread of NCLC1 between cells in a diatom filament (Figure S2), (4) release of NCLC1 cells or a secondary infection (Figures 3D–3F), and (5) NCLC1 multinucleate phase (Figures 3A–3C).

with three independent counts. These analyses consistently demonstrated that \sim 2%–8% of the FISH identified cells were indicative of intracellular infections of diatoms (Figures 4A and 4B; Table S2).

Counterstaining of DNA with propidium iodide demonstrated that the FISH probe staining often surrounded a DNA structure. In several of the images, the diatom nucleus was not visible within the infected cell as a separate entity (Figure 2A, 2B, 2E, and S2), yet in some cases the diatom nucleus was observed in uninfected diatom cells residing next to the NCLC1-infected cell within the diatom filament (Figures 2E and S2). These microscopy results suggest that the DNA structure identified is either the NCLC1 nucleus/nuclei and that the diatom nucleus is absent, suggesting, in some cases, that the NCLC1 association is with a diatom carcass, or, alternatively, that the host diatom nucleus is actually surrounded by the infecting NCLC1 cell(s) (see Figures 2B, 2D, 2E, and S2, with further rationale outlined in the Figure 2 legend). Either characteristic implies a parasitic interaction; however, we note that such analyses are complicated by the FISH process of sampling, which can damage cells, and the limitations of microscopy, which cannot completely account for cellular

structures throughout the z axis of the microscopic field. These limitations also prevent quantitative comparisons. Nonetheless, these results are consistent with the hypothesis that NCLC1 cells are present within dead diatom cells or diatom cells with nuclei smothered by the NCLC1 infection.

In addition to the intracellular associations identified, the FISH analysis demonstrated a large proportion of candidate NCLC1 cells positioned proximate to a diatom cell surface, suggesting an epibiotic (surface-to-surface) association. Three diatom genera were detected in high cell concentrations: *Chaetoceros*, *Skeletonema*, and *Pseudonitzschia* (Table S1; Figure S3). From our FISH micrographs, we inferred the taxonomic affiliation of the NCLC1-bearing diatoms based on the bright-field silhouette present on the filter. These results indicate that the NCLC1 association was present across a range of diatom hosts, including members from the most abundant diatom genera identified (i.e., *Chaetoceros*, *Skeletonema*, and *Pseudonitzschia*). We note that the pattern of epibiotic associations was similar for both surface and DCM water-column samples (\sim 30%; see Table S2; Figures 4A and 4B), consistent with the detection of similar patterns of phytoplankton biodiversity present in both samples (Figure S3; Table S1), a result that suggests that the surface and DCM zones were highly homogeneous in terms of diatom species community composition.

Although this putative extracellular association and, indeed, the intracellular associations discussed above suggest a symbiotic-parasitic interaction between NCLC1 and diatoms, these

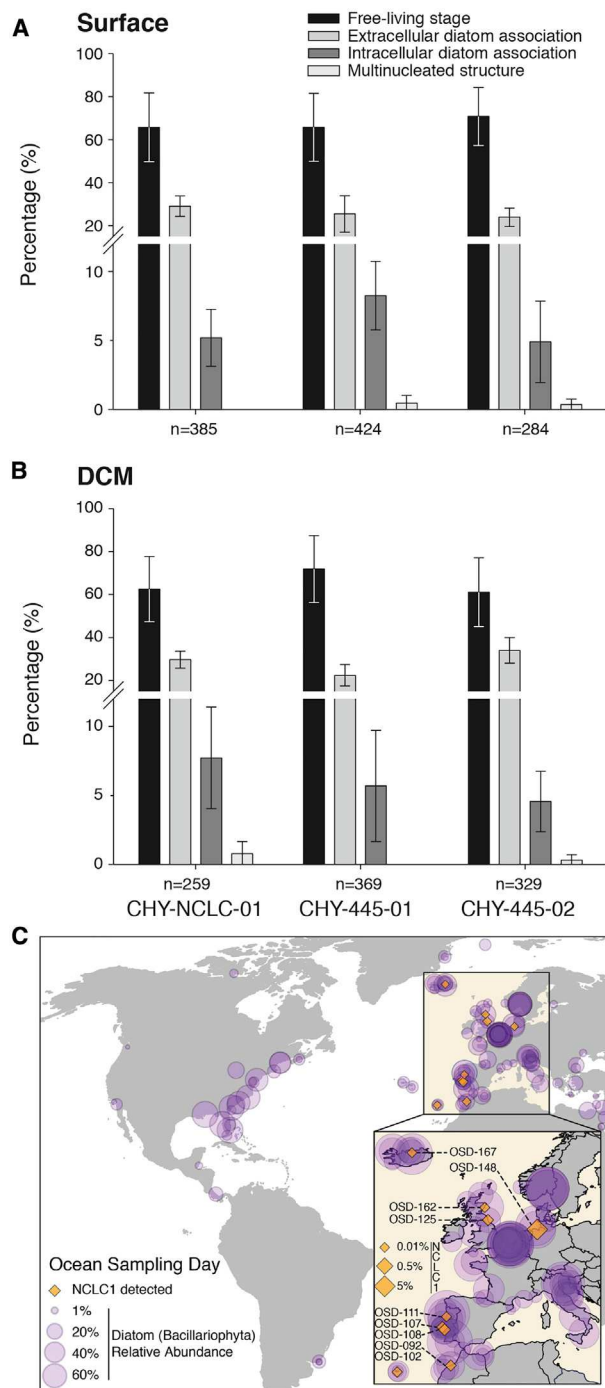


Figure 4. Detection Provenance of NCLC1

(A and B) Percentage of different cell types recovered in the FISH analysis from (A) Sub-surface and (B) DCM depths. The different probes used are listed across the x axis of (B). The total number of NCLC1 cells identified using each probe is also listed below the x axis. Mean % shown is derived from independent hybridization experiments conducted on three separate filter pieces per probe. In each case, three independent counts were conducted per filter piece with a minimum of 200 FISH positive cells observed. Error bars indicate the standard deviation. See Table S2 for data.

(C) Geographical distribution of NCLC1 and diatoms across the Ocean Sampling Day (OSD) data. Samples where SSU-V4 amplicon sequence variants (ASV) classified as NCLC1 and diatoms (Bacillariophyta) were detected are

observations could also be the product of filtration, which artifactually, but consistently, suggests a physical contact between abundant diatom species and NCLC1 cells. To test if the observed NCLC1-diatom extracellular associations were a filtration artifact, we used conservative Bonferroni-corrected exact binomial tests with a minimal hypothesized association parameter (0.01). This resulted in rejection of a null hypothesis of minimal chance interaction between NCLC1 and *Chaetoceros* spp. and between NCLC1 and *Pseudonitzschia* spp. (both intracellular or extracellular diatom associations were counted), regardless of probe used (Figure 2G). Collectively, these data suggest that the NCLC1 group detected is forming a bona fide cellular association with multiple diatom species.

To further explore this association, we used the publicly available 2014 Ocean Sampling Day citizen science [41] projects' SSU V4 rRNA gene sequences to examine the co-occurrences of NCLC1 amplicon sequence variants (ASVs) with other eukaryotic ASVs (see Table S3). These data demonstrate that the detection of NCLC1 was geographically restricted to the North-East Atlantic and the North Sea (Figure 4C). This constitutes an incomplete and biased sampling profile, but it suggests that NCLC1 is resident in these environments but is absent and/or undetected from water samples on the western side of the Atlantic on the date of sampling. This analysis identifies a relatively low abundance for NCLC1, except for one sample (OSD-148), which demonstrated an ~4% relative abundance of an NCLC1 SSU rRNA gene sequence (Figure 4C and Table S3). We identified three significant NCLC1 co-occurrences within the Ocean Sampling Day data; one association was between an NCLC1 ASV and a rhizarian ASV, although we note that sampling of this ASV within the Ocean Sampling Day dataset had limited reads and so may be an artifact, even though a significant pseudo p value was recovered (Table S3). More convincingly, and consistent with the FISH identification of a NCLC1-diatom association reported here, we identified two significant co-occurrence patterns between an NCLC1 and a diatom ASV, providing further support for this interaction across geographically distributed sites (Figure 4C; Table S3).

Detection of Additional NCLC1 Life-Cycle Stages

The FISH experiments also detected two additional variant cell types that were absent from the negative controls and were not associated with diatoms or an identifiable second-party cell, suggesting detection of free-living forms of the target NCLC1 group. This alternative cell type was detected in the 3–20 μm filtrate and the 20–1,000 μm plankton net samples. Using both specific and general NCLC1 probes, we observed a multi-nucleated structure (Figures 3A–3C). This structure was rarely seen in our samples but was detected independently using all three probes and from samples recovered from both sub-surface and DCM water fractions. Specifically, we identified 0%, 0.47% ($\pm\text{SD } 0.55$), and 0.35% (± 0.41) of all the FISH-detected cells from the sub-surface water samples and 0.77% (± 0.89),

indicated by orange diamonds and purple circles, NCLC1 and diatoms, respectively. Diamond and circle marker sizes are scaled according to NCLC1 and diatom relative abundance recovered (see key). See also Table S3.

0%, and 0.3% (± 0.41) of all the FISH-detected cells from the DCM water samples. These data are consistent with the hypothesis that the putative NCLC1 cells detected form a multinucleate sporangium-like reproductive life-cycle stage. The low detection rate suggests that this form is rare and/or short-lived; alternatively, the process of fixation or filtration may have disrupted these multicellular life-cycle stages, making them difficult to recover.

The second cell type observed was ovoid (length = $7.70 \pm 1.08 \mu\text{m}$, width = $3.72 \pm 0.55 \mu\text{m}$, $n = 20$) or round (diameter = $6.73 \pm 0.58 \mu\text{m}$, $n = 20$). These cellular structures are likely to correspond to either a spore or a cyst life-cycle stage (Figures 3D–3F) or alternatively they may represent an association with a yet- unidentifiable second host. We identified a high proportion of these putative life-cycle-stage cells (60%–70%; see Figures 4A and 4B) from both sample depths using the general and specific NCLC1 probes. CFW staining of the filters coupled with FISH microscopy demonstrated that all cell types identified did not possess a detectable cellulose and/or chitin cell wall; however, we note that the cells sampled possibly represent only a fraction of the NCLC1 life cycle.

Conclusions

These results demonstrate a hitherto-undetected intracellular infection of diatoms, an ecologically important group of marine phytoplankton, including *Chaetoceros*, *Skeletonema*, and *Pseudonitzschia* species, that can form blooms in marine waters. The host range also includes groups responsible for harmful algal blooms (e.g., *Pseudonitzschia* spp.). The infectious agent constitutes a phylogenetically unique branch—most likely a distinct and diverse addition to the Holomycota and possibly the Opisthosporidia [7]—adding an additional branch close to the base of the radiation of the fungal kingdom. The nature of the NCLC1-diatom interaction is unknown but potentially represents a parasitic infection, a mutualistic interaction, a saprotrophic degradation of dead diatoms, or, indeed, an infection that transitions between all three modes of interaction. NCLC1 DNA- and RNA-derived sequences have been detected in the marine water column and sediments, suggesting that NCLC1 is active in both pelagic and benthic environments [4]. As such, the NCLC1 cells may follow diatom carcasses into the marine sediment as saprotrophic degraders of these phytoplankton cells. Consistent with this later hypothesis, we observe NCLC1 as an intracellular infection within diatoms with no identifiable nuclei next to diatoms with identifiable nuclei, consistent with the hypothesis that the host is dead and/or that the diatom nucleus has been smothered by NCLC1 cells. We therefore suggest that this interaction represents a necrotrophic-parasitic interaction followed by a saprotrophic interaction with *Chaetoceros*, *Skeletonema*, and *Pseudonitzschia* diatom carcasses. As such, NCLC1 joins an increasing list of viral (e.g., [42, 43]), protist [2, 24, 25], and fungal pathogens, including putative chytrid associations [44, 45], which are hypothesized to infect diatoms and determine the fate of important phytoplankton blooms.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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- QUANTIFICATION AND STATISTICAL ANALYSIS
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 - SSU sequence alignment and phylogenetic tree reconstruction
 - Ocean Sampling Day 2014 and sequence co-occurrence analysis
- DATA AND CODE AVAILABILITY

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.cub.2019.09.074>.

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AUTHOR CONTRIBUTIONS

A.C., A.M., and T.A.R. conceived and designed the study. A.C. conducted FISH Microscopy, A.M. and A.C. conducted phylogenetic and Ocean Day sampling analysis, and F.M. designed and conducted statistical analysis. B.E. and W.E. conducted marine sampling and conducted plankton counts. All authors contributed to drafting the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Tat1 Tubulin Antibody	Gift K. Gull Uni. Oxford, By request	TAT1; RRID: AB_10013740
Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulins	Jackson ImmunoResearch/ Strattech	https://www.jacksonimmuno.com/catalog/products/115-095-003
Biological Samples		
Filter sections taken as part of the BioMarks project	Biomarks Consortium, by request, but exhaustible.	N/A
Chemicals, Peptides, and Recombinant Proteins		
Calcofluor White	Sigma-Aldrich, USA	https://www.sigmaaldrich.com/catalog/product/sial/18909?lang=en&region=GB
Deposited Data		
All physical and chemical parameters of the samples water column obtained using a CTD	This paper	are available at http://biomarks.eu/ctd007 (and replicated at FigShare https://doi.org/10.6084/m9.figshare.9821936)
tree file, masked and unmasked SSU rDNA alignments	This paper	Zenodo repository: https://doi.org/10.5281/zenodo.2788876 .
Ocean Sampling Day 2014 Data	[41, 46]	https://github.com/MicroB3-IS/osd-analysis/wiki/Guide-to-OSD-2014-data see also http://mb3is.megx.net/osd-files?path=/2014/protocols
Oligonucleotides		
FISH Probe (5'-3') GTCCTAGATTCAGTCTC	This paper, ordered from biomers.net (Germany)	CHY-NCLC-01
FISH Probe (5'-3') GATTCTAATGCCCCCAA	This paper, ordered from biomers.net (Germany)	CHY-445-01
FISH Probe (5'-3') CGATTCTAATGCCCCCA	This paper, ordered from biomers.net (Germany)	CHY-445-02
FISH Probe (5'-3') [reverse complement negative control] GAGCTGTGAATCTAGGAC	This paper, ordered from biomers.net (Germany)	CHY-NCLC-01_RC
FISH Probe (5'-3') [reverse complement negative control] TTGGGGGCATTAGAATC	This paper, ordered from biomers.net (Germany)	445_01_RC
FISH Probe (5'-3') [reverse complement negative control] GTTGGGGGCATTAGAAT	This paper, ordered from biomers.net (Germany)	445_02_RC
Software and Algorithms		
The R code used to test statistical association between NCLC1 and Diatoms in the FISH data	This paper	Zenodo repository: https://doi.org/10.5281/zenodo.2788876 .
mathFISH for FISH probe design	[47]	http://mathfish.cee.wisc.edu
ARB software (v.6.0.4) for SSU rRNA probe design	[48]	(https://www.arb-silva.de)
TESTPROBES for FISH probe optimization	[48]	(https://www.arb-silva.de)
R programming language (RCore2013) 'tidyverse' set of tools for statistical analysis	N/A	(https://www.tidyverse.org/)
BLASTN similarity search for sequences with shared sequence identify from NCBI nt database	[49]	https://blast.ncbi.nlm.nih.gov
MAFFT v7.2 sequence alignment for automated sequence alignment	[50]	https://mafft.cbrc.jp/alignment/server/
trimAL v4 for automated sequence alignment refinement and sampling	[51]	http://trimal.cgenomics.org

(Continued on next page)

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
seaview v4 manual sequence alignment program	[52]	http://doua.prabi.fr/software/seaview
IQ-TREE v1.6 for phylogenetic analysis	[53]	http://www.iqtree.org/release/v1.6.7
ModelFinder for finding appropriate model of sequence evolution for phylogenetic analysis	[54]	http://www.iqtree.org/ModelFinder/
PHYLOBAYES v3.3 for Bayesian phylogenetic analysis	[55]	http://megasun.bch.umontreal.ca/People/lartillot/www/download.html
DADA2 for sequence tag analysis	[56]	https://benjjneb.github.io/dada2/dada-installation.html
R package 'ggmap'	[57]	https://github.com/dkahle/ggmap
SparCC	[58]	https://bitbucket.org/yonatanf/sparcc
Other		
PR ² v4.10 SSU rDNA reference database	[59]	https://github.com/pr2database/pr2database

LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact Thomas Richards (T.A.Richards@exeter.ac.uk). This study did not generate any unique reagents other than the FISH oligonucleotide probes. Details of these probes are available in the key resource table.

EXPERIMENTAL MODEL AND SUBJECT DETAILS**Sampling**

Samples were taken as part of the BioMarkS project (<http://www.biomarks.eu>) [37] in the outer Oslofjorden station OF (59.253735N, 10.710908E) on the 22nd September 2009. All physical and chemical parameters of the water column obtained using a CTD are available at <http://biomarks.eu/ctd007> (and replicated here DOI: [10.6084/m9.figshare.9821936](https://doi.org/10.6084/m9.figshare.9821936)). Water and plankton samples were collected from sub-surface at 1 m depth and the DCM at 20 m depth from the University of Oslo research vessel R/V Trygve Braarud. Sampling was conducted using either: 1) a plankton net with 20 μm mesh-size, for a horizontal net haul where the net sample was then passed through a 1000 μm metallic sieve, or 2) Niskin bottles for collecting water samples. Aliquots from all samples were fixed onboard with neutralized formaldehyde (3.7% final conc.) and kept at 4°C until processed in the lab the day after. In the lab the samples from the plankton net (with a 20 μm 'aperture' size) were collected onto a 47 mm polycarbonate (PC) filters of 12 μm pore-size rendering recover of cells of 20 - 1000 μm diameter. For the Niskin bottle samples, water was pre-filtrated through 20 μm nylon sieve and then successively size-fractionated throughout 3 μm and 0.8 μm PC filters of 25 mm diameter. All filters (including plankton net water samples of 20- 1000 μm and water samples of 3- 20 μm and 0.6- 3 μm size fractions) were then dehydrated in sequential 50%, 80% and 100% ethanol incubations with 3 min of incubation at each step followed by drying at room temperature before final storage at -80°C .

METHOD DETAILS**Phytoplankton counts**

Cell counts was performed on 10 mL water samples collected by the Niskin bottles and fixed immediately with Lugol's solution (1% final. conc.). Cells were counted using the Utermöhl method [60, 61].

Probe design

FISH probes were designed based on an alignment of 136 sequences and 316 alignment positions of the V4 region of the SSU rRNA encoding gene (see [4]) using the probes-design tool available through ARB v6.0.4 [62]. We designed three oligonucleotide probes, a general probe targeting the wider NCLC1 clade named CHY-NCLC-01 and two probes, CHY-445-01 and CHY-445-02, which specifically target the cluster 445 (see [Key Resources Table](#)). The thermodynamic parameters for all three probes were evaluated using mathFISH [47]. For the two specific probes of the cluster 445, only one specific region of the V4 SSU rDNA contained enough nucleotide polymorphism to allow design of highly specific probes with the optimal thermodynamic properties. We therefore decided to use two different probes with a single shift in the nucleotide sequence position (i.e., CHY-445-01 and CHY-445-02).

For negative controls for the specific and general probe we used the reverse complement of each probe named CHY-445-01-RC, CHY-445-02-RC and CHY-NCLC-01-RC (see [Key Resources Table](#)) and the hybridization buffer without any probe. All probes were

tested *in silico* using both ARB software (v.6.0.4) and TESTPROBES available on the Silva website (<https://www.arb-silva.de>) [48]. The six oligonucleotide probes were purchased from biomers.net (Germany) and were labeled at 5' end with horseradish peroxidase (HRP).

Fluorescent *in situ* hybridization

For *in situ* hybridization, we followed the protocol described by Chambouvet et al. 2008 [18]. This method is outlined as follows: filter samples prepared for FISH were incubated with 3 μL of probes (10 pmol L^{-1}) and 27 μL of hybridization buffer (HB) that include 35% (v/v) formamide, 0.9 M NaCl, 20 mM TrisBase pH = 7.5, 0.01% SDS (sodium dodecyl sulfate, Sigma-Aldrich, UK) and 2% blocking reagent. Samples were hybridized for 12 h at 35°C or 42°C depending of the probe used (see [Key Resources Table](#)) before washing twice at 46°C during 20 min in a washing buffer (56 mM NaCl, 5 mM EDTA, 0.01% SDS, 20 mM Tris HCl pH = 7.5). Filter samples were then equilibrated for 15 min at room temperature in the dark in TNT buffer (100 mM Tris-HCl pH = 7.5, 150 mM NaCl, 0.05% (v/v) Tween 20 (Sigma Aldrich, UK)). Each filter was then transferred onto a new slide before adding 10 μL of TSA mix (TSATM Fluorescein System, Perkin Elmer, UK) per filter piece (2 μL of FT, 50 μL of amplification diluent and 50 μL of 40% dextran sulfate) and incubated for 30 min at room temperature in the dark. To remove excess TSA amplification, samples were incubated twice at 55°C for 20 min in TNT buffer. Filters were then washed twice in sterile water and left dry at room temperature. Finally, filter samples were mounted between a slide and a cover glass using an anti-fade mounting solution AF1 (CitifluorTM, Electron Microscopy Science, USA) previously mixed with DNA counterstaining, propidium iodide (final concentration of 10 $\mu\text{g/ ml}^{-1}$). Counts were performed with a Zeiss Observer Z1. Each picture was obtained from a single image extract from a Z stack using Zeiss Observer Z1 epifluorescence microscope equipped with a 3D module VivaTome, a laser excitation light and a camera AxioCam MR. All FISH experiments were conducted in triplicate for each sample type and each experimental condition.

To detect flagellum structures, we used antibodies and the protocol reported in [35] to identify major tubulin cytoskeleton of flagella. Briefly to ascertain presence of a flagellum, sections of filter that were subjected to TSA-FISH hybridization were re-permeabilized with 0.1% v/v nonidet P-40 in PBS (10 mM Na₂HPO₄, 2 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl, pH 7.2), blocked with 1% w/v bovine serum albumin in PBS then incubated for 1 h with the TAT1 monoclonal antibody [63] against α -tubulin, followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulins (Jackson ImmunoResearch/Strattech). The antibody was a gift from Professor Keith Gull's lab at the university of Oxford. Across all samples, candidate flagella seemed dissociated from cells, this was specifically apparent in the dinoflagellates, where the flagella seemed sheared off, indicating that the fixation and dehydration steps were too rough to perform this cellular structure assay. Therefore we could not reliably assess NCLC1 cells for candidate flagella.

To detect cellulosic and/or chitin cell wall structures, we stained with Calcofluor White (1% final concentration, Sigma-Aldrich, USA) using the protocol reported in [35]. We also used this protocol to check for NCLC1 associations with additional cellulosic and/or chitin containing host cells such as dinoflagellates, which were also present in the environmental samples ([Table S1](#)). This check was conducted to rule out additional host associations among cells damaged during the process of cell sampling and FISH microscopy preparation, which could potentially limit our ability to identify host cell morphology.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical testing of NCLC1-diatom associations

In order to test whether each observed NCLC1-diatom interaction was significantly different from an incidental interaction due to filtering artifacts we used a series of 1-sided binomial tests ($\alpha = 0.05$) using a binomial distribution with a minimal theta (0.01). In other words, each observed interaction was treated as a 'success' with the total number of NCLC1 interactions observed (i.e., the row sum) as the total number of trials. In order to control for the multiple comparisons a Bonferroni correction was applied to the results. The boxplots were generated to show the distribution of different interactions by summing the replicates per slide. Plots were ordered by their median values and interactions where the null hypothesis of a minimal interaction was rejected were highlighted in blue. This analysis was conducted in the R programming language (RCore2013) using the 'tidyverse' (<https://www.tidyverse.org/>) set of tools. The code used to perform these calculations (plotting_and_testing_association.r) can be found in the supplemental data, see Zenodo repository: DOI [10.5281/zenodo.2788876](https://doi.org/10.5281/zenodo.2788876).

SSU sequence alignment and phylogenetic tree reconstruction

SSU sequences from 200 taxa were retrieved from previous publications [4, 6]; additional sequence homologs were identified through BLASTN similarity searches (<http://blast.ncbi.nlm.nih.gov/blast.ncbi.nlm.nih.gov/>). Sequences were then aligned using MAFFT v7.2 iterative refinement method Q-INS-i (<https://mafft.cbrc.jp/alignment/server/>, [50]). The alignment was subsequently edited with trimAL v4 [51] using the 'gappyout' parameter ("distribution0based algorithm") and manually masked with seaview v4 [52], resulting in a final alignment of 1542 nucleotide sites (of which 1221 were parsimony informative). The ML phylogenetic tree was reconstructed with IQ-TREE v1.6 [53] using a thorough nearest neighbor interchange search ('-allnri') under the GTR+F+R6 model that is, the general time reversible model with empirical base frequencies and FreeRate model [64] with 6 categories; this substitution model was determined as best fitting the data by ModelFinder [54], as implemented in IQ-TREE, and based on the Akaike Information Criteria. To evaluate node supports, 100 nonparametric bootstrap trees were reconstructed using the same methodology. In addition, to the ML reconstructions, Bayesian inferences were conducted with PHYLOBAYES v3.3 [55] under the GTR-CAT- Γ model

(with 4 discrete categories). Two independent Markov chain Monte Carlo chains (MCMC) were run for 20k generations and sampled every 10 generations with the first 3k discarded as the burnin. Resulting posterior probabilities were mapped onto the ML phylogeny presented.

Ocean Sampling Day 2014 and sequence co-occurrence analysis

SSU rDNA amplicon sequence and contextual data from the Ocean Sampling Day 2014 initiative [41] were retrieved from the Micro B3 project repository (<https://github.com/MicroB3-IS/osd-analysis/wiki/Guide-to-OSD-2014-data>); microbial community filtering, DNA extraction and Illumina MiSeq sequencing protocols are described in the OSD handbook (<http://mb3is.megx.net/osd-files?path=/2014/protocols>; see also [46] for an overview of the sampling and sequencing protocol). OSD amplicon sequences were retrieved as pre-processed sequences (technical sequences removed; non-merged paired end reads) and only SSU-V4 sequences obtained following the NPL022 protocol (according to the OSD nomenclature) were analyzed as part of this study. To allow comparison between depth samples, only samples collected from surface waters were kept for analysis. All OSD amplicon were then processed with DADA2 [56]; SSU-V4 sequences were first error corrected and dereplicated, and paired end reads were merged to produce an amplicon sequence variant (ASV) dataset. ASV sequences were then checked for potential chimeras. ASVs were taxonomically classified using the PR² v4.10 reference database ([59]; <https://github.com/pr2database/pr2database>) and the RDP naive Bayesian classifier [65] using a minimum bootstrap confidence of 50, as implemented in DADA2. To produce an SSU-V4 dataset representing only protists sampled during OSD-2014, ASVs classified as multicellular eukaryotes were discarded from the study. Furthermore, to detect spurious SSU-V4 sequences, ASVs were aligned to PR2 representatives using MAFFT v7 [66] and ASVs with poor sequence overlap thresholds, identified with trimAl v1.4 ([51]; '-seqoverlap' lower than 0.8), were discarded. The final SSU-V4 sequence dataset was comprised of 7,766 ASVs totalling 2,552,000 sequences across 145 samples. ASV geographical distributions were plotted using the R package 'ggmap' [57]. ASV sequence correlations were determined for ASV represented by at least 20 sequences (to limit computation time and potential spurious correlations) using the 'sparse correlations for compositional data' algorithm, SparCC [58], with 20 iterations; to identify significant co-occurrences, pseudo p values were computed from resampled correlation matrices (i.e., 100 bootstrap replicates).

DATA AND CODE AVAILABILITY

All data and code are available with DOI's given in the methods section. Specifically, physical and chemical parameters of the water column obtained using CTD ocean water sampling are available at <http://biomarks.eu/ctd007> (and replicated here at figshare DOI: [10.6084/m9.figshare.9821936](https://doi.org/10.6084/m9.figshare.9821936)). The phylogenetic tree file, masked and unmasked SSU rDNA alignments are available at Zenodo repository: DOI [10.5281/zenodo.2788876](https://doi.org/10.5281/zenodo.2788876). All sequence data used were derived from the NCBI 'GenBank' database and accession numbers are provided in Figure 1B. The R code used to test statistical association in the FISH data are available at Zenodo repository: DOI [10.5281/zenodo.2788876](https://doi.org/10.5281/zenodo.2788876).

Titre : Ecologie fonctionnelle des Perkinsozoa (Alveolata) en milieu marin : de la diversité globale à l'étude d'un cas de co-infection entre *Perkinsus olseni* et *Perkinsus chesapeaki*.

Mots clés : Perkinsozoa, *Perkinsus*, micro-parasites eucaryotes, diversité environnementale, co-infection, palourde japonaise.

Résumé : Les microparasites eucaryotes sont une composante essentielle des écosystèmes, dont l'influence est considérable sur la dynamique des populations hôtes et le fonctionnement des réseaux trophiques. Le clade des Perkinsozoa (Alveolata), composé de quatre grands groupes parasitaires, en est un exemple représentatif en milieu marin. Certains de ces membres emblématiques, comme *Perkinsus* spp., représentent de réelles menaces écologiques et économiques en raison de leur caractère invasif, de leur pathogénicité et de leur grande plasticité phénotypique. L'objectif de cette thèse était de caractériser le rôle écologique et fonctionnel des Perkinsozoa d'abord à une échelle globale puis à une échelle populationnelle. Au sein de l'Océan globale, le groupe des Perkinsozoa est diversifié (109 ASVs – 6 clusters), peu abondant (<1%) et très localisé dans les communautés picoplanctoniques pélagiques. Un nouveau cluster actif, le Cluster A, a été mis en évidence dans la zone mésopélagique.

Cependant, il est impossible de déterminer si ces Perkinsozoa putatifs sont symbiotiques ou libres car aucun d'eux n'a été caractérisé à ce jour. A l'échelle des populations, une méthodologie de PCR quantitative en temps réel en duplex a été élaborée pour l'étude *in situ* de la co-infection entre *Perkinsus olseni* et *P. chesapeaki* au sein d'une population de palourdes japonaises, *R. philippinarum*, dans le bassin d'Arcachon. Cette coinfection, répandue dans le bassin, représente 12 à 36 % des palourdes échantillonnées tandis que la monoinfection à *P. olseni* reste majoritaire. La présence sporadique de *P. chesapeaki* au sein de certains organes hôte supposent que cette infection serait secondaire et pourrait être influencée par des paramètres environnementaux jouant sur les stades du cycle de vie parasitaire. Dans un contexte global d'émergence des maladies, une vision intégrative des relations hôtes-parasites est nécessaire pour résoudre le rôle écologique des Perkinsozoa dans différents écosystèmes

Title : Functional ecology of Perkinsozoa (Alveolata) in marine environments : from global diversity to the study case of co-infection between *Perkinsus olseni* and *Perkinsus chesapeaki*.

Keywords : Perkinsozoa, *Perkinsus*, eukaryotic micro-parasites, environmental diversity, co-infection, Manila clam.

Abstract : Eukaryotic microparasites are essential component of ecosystems and have a considerable influence on host population dynamics and food web functioning. The Perkinsozoa lineage (Alveolata), composed of four groups of described parasitic protists, is a characteristic study case in marine environment. Some of them, e.g. the molluscan parasites *Perkinsus* spp., constitute serious ecological and economic threats because of their invasive character, pathogenicity and high phenotypic plasticity. The aim of this work was to characterise the ecological and functional role of Perkinsozoa at global and population scales. Within the global Ocean, Perkinsozoa clade is diverse (109 ASVs - 6 clusters), low in abundance (< 1 %) and very localised in pelagic picoplanktonic communities. A new ribosomally active cluster, Cluster A, has been identified in the mesopelagic zone from Pacific, Atlantic and Indian oceans.

However, it was not possible to determine whether these putative organisms are symbiotic or free-living because none of these have been characterised so far. At the population level, a duplex real-time quantitative PCR methodology has been developed for the *in situ* study of co-infection between *Perkinsus olseni* and *P. Chesapeaki* in Manila clam, *Ruditapes philippinarum*, population from the Arcachon bay (France). This co-infection is widespread in this area, ranging from 12 to 36% of the clams sampled. Global infections are mainly dominated by mono-infection with *P. olseni*. Sporadic occurrence of *P. chesapeaki* in specific host organs suggests that this infection is secondary and could be influenced by environmental parameters affecting stages of the parasitic life cycle. In a global context of emerging diseases, an integrative view of host-parasite relationships is needed to resolve the ecological role of Perkinsozoa in different ecosystems.