The Infection Cycle of *Marteilia pararefringens* sp. nov. in the blue mussel, *Mytilus edulis* L.

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Abstract

In 2016 the first case of marteiliosis in Norway was reported by the Institute of Marine Research. The discovery occurred in Agapollen, Bømlo, a previously active oyster pond. The parasite *Marteilia pararefringens* sp. nov. was found to only infect mussels, *Mytilus edulis*, and not the cohabiting flat oysters, *Ostrea edulis*. In May 2018, 250 naïve mussels, *Mytilus edulis*, were deployed in a channel connected to the oyster pond to examine the transmission period and progression of *Marteilia pararefringens* in the host. In October 2018, another 250 naïve mussels were deployed to examine whether transmission could occur during sporulation of the parasite from the mussel host. Sampling of 30 mussels for PCR and histological analysis every six weeks. The transmission period was found to occur from July, based on PCR results. The first mussels were infected in August based on histological evidence. All target tissues (stomach epithelium, ducts, and digestive tubules) were infected at this time. Sporulation occurs from August to November, evidenced by the presence of advanced stages in digestive tubules and sporonts in the lumen of the digestive tract. The spread of the infection involves an exponential increase of the number of parasites per digestive tubule. The infection of *M. pararefringens* triggers a host haemocyte reaction associated with the infection intensity. Focal necrosis and degeneration of digestive tubules is often seen. Sporulation occurs in conjunction with the disruption and physiological dysfunctions of the digestive tubules, potentially leading to the death of the host.

The second group (October to March) did not become infected. A cohabitation trial in the laboratory to see whether the parasite could infect healthy mussels (n = 100) from donor mussels (n = 50) was unsuccessful, indicating the need for an intermediate host. Plankton samples were PCR positive from July to October, coinciding with the transmission period of the parasite to the mussels. Therefore, the intermediate host could potentially be of planktonic origin.

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Abbreviations

RNA	Ribose Nucleic Acid
DNA	Deoxyribose Nucleic Acid
IMR	Institute of Marine Research
UiB	University of Bergen
OIE	World Organization for Animal Health
NDT	Non-infected Digestive Tubules
IDT	Infected Digestive Tubules
PIDT	Percentage of Infected Digestive Tubules
ES	Early stage of sporulation
IS	Intermediate stage of sporulation
AS	Advanced stage of sporulation
PES	Percentage of early stage of sporulation
PIS	Percentage of intermediate stage of sporulation
PAS	Percentage of advanced stage of sporulation
CEFAS	Centre for Environment, Fisheries and Aquaculture Science
SSU rRNA	Small Subunit ribosomal Ribose Nucleic Acid
RFLP	Restriction Fragment Length Polymorphism
MNPIDT	Mean number of parasites per infected digestive tubule
PCR	Polymerase Chain Reaction
HES	Hematoxylin Eosin Saffron staining
DT	Digestive tubule
HHR	Host Haemocytic Reaction
PMCHI	Percentage of mussels in each category of haemocytic infiltration
UIB	Unknown Intracellular Bodies
FET	Fisher Exact Test
ANOVA	Analysis Of Variance test
ANCOVA	Analysis Of Covariance test

1 Introduction

1.1 Aquaculture on a Global Scale

The aquaculture industry has grown at an impressive rate over the past decades and is now a major contributor to global food production. Since 2000, aquaculture contribution in the market has increased from 25.7% to 46.8% in 2016 (FAO 2018). The industry is believed to play a vital role in sustaining a growing world population (FAO 2018).

On a global basis, molluscs represent the second most important subgroup in terms of production volume (17.1 million tonnes, 2016), outmatched only by inland aquaculture of finfish (47.5 million tonnes, 2016) (FAO 2018). In marine and coastal aquaculture, molluscs represent 58.8% of total production, dwarfing the contribution of market favourites such as Atlantic salmon (*Salmo salar*) (FAO 2018).

Even though total production values of molluscs are far higher than other marine species on a global scale, their economic relevance is comparatively smaller. In EU, molluscs represented 47% of the total production volume of 1.25 million tonnes in 2014, compared to diadromous fish (including salmon and rainbow trout, *Oncorhynchus mykiss*) at 29.7% (Eurostat 2016). The production value in the same period, however, was 23.3% and 37.8%, respectively (Eurostat 2016). Only a handful of countries tend to focus their production on shellfish, where the environment is favourable and professional expertise is prevalent, such as France and Spain (Eurostat 2016).

In terms of gross mass production, Mediterranean mussels (*Mytilus galloprovincialis*) and blue mussels (*Mytilus edulis*) account for roughly 470 thousand tonnes (more than a third) (Eurostat 2016). In Spain, *M. galloprovincialis* accounts for 77% of the live weight of all species cultured in this region (Eurostat 2016). In France, the Pacific oyster (*Crassostrea gigas*) represent 38% of the total production, followed by *M. edulis* at 30%, and a few *M. galloprovincialis* production sites, responsible for 7% (Eurostat 2016).

Even though different bivalve species are mainly grown in specific regions (e.g. *M. galloprovincialis* in Spain, *C. gigas* in France, etc.), this pattern is non-static, meaning it can change based on economical, ecological and, more applicably, disease factors that can affect

the local populations (I. Arzul 2018). For example, the overexploitation of natural reservoirs of the flat oyster *Ostrea edulis* in the late 1800s in France prompted the importation the cupped oyster, *Crassostrea angulata*, from Portugal (Goulletquer & Heral 1997; Roch 1999). This revitalised the production of oysters in this region, with both species contributing equally to the total production volume in the 1900s (Roch 1999). Massive mortalities of *O. edulis* by an unknown cause in the 1920s favoured the further production of *C. angulata* for a limited time, when the latter species disappeared due to an iridovirus infecting the gills. Gill disease eradicated *C. angulata* along the French coast during 1967-1973 (Goulletquer & Heral 1997; I. Arzul 2018). Once again in an effort to rejuvenate the oyster production in France, farmers imported spat and adults of the Pacific oyster, *Crassostrea gigas* (Grizel and Héral, 1991). This stock reinforcement was successful and facilitated a fast industry recovery (Goulletquer & Heral 1997). Then, the spread of two diseases, marteiliosis caused by the paramyxean parasite *Marteilia refringens* and bonamiosis caused by the haplosporidian parasite *Bonamia ostreae*, decimated the production of *O. edulis* in almost all rearing areas (Goulletquer & Heral 1997). Despite efforts to restock, produce resistant oysters and alter management strategies, the production of flat oysters has remained relatively low since (Goulletquer & Heral 1997).

The aquaculture of the robust Pacific oyster sustains the production of oysters in much of Europe, but even this species could collapse at the outbreak of disease (Goulletquer & Heral 1997; Murray et al. 2012; I. Arzul 2018). A variant of the oyster herpesvirus, OsHV-1µvar, has already shown detrimental effects on stocks in France (Segarra et al. 2010) and has been detected from oysters in Australia, England, Ireland, Mexico, New Zealand, US (Arzul et al. 2017; Murray et al. 2012), and Norway (Mortensen et al. 2016). The transport of infected spat to regions free of the virus could have massive economic implications. Potentially, this could cripple the oyster aquaculture industry to a degree difficult to bounce back from, echoing the case with *C. angulata* and *O. edulis* (Arzul 2018; Arzul et al. 2017; Murray et al. 2012).

1.2 Vulnerability of Molluscs

With the sheer volume of shellfish produced in Europe and worldwide, it is important to maintain tight control of the health and disease surveillance programs to restrict potential epidemics and mass mortalities. However, this is no simple task.

Mussels and oysters are filter feeders; they feed on plankton, other microscopic organisms, and particulate organic material that is found in seawater. This means that they are in direct contact with a plethora of potential pathogens at any given time. The only defence mechanism shellfish have against these microorganisms is the innate immune system, incapable of memory following the first encounter with a pathogen (Tiscar & Mosca 2004).

The invertebrate immune system is paramount to resistance against infection, and in the case of parasites, either by elimination or segregation (Roch 1999). Considering invertebrates far predate vertebrates, the defence system is palpably effective despite the lack of potent acquired immunity extant in the latter (Roch 1999). The innate immune system is limited to circulating molecules (humoral immunity) and circulating cells (cellular immunity), with differing effectiveness dependent on the invading body (Roch 1999). The humoral system is composed of macromolecules found in the extracellular fluids or produced by haemocytes, such as agglutinins, antimicrobial peptides, lectins, and lysosomal enzymes (Tiscar & Mosca 2004). Though it undeniably plays an important part in combating foreign bodies, there is no evidence that the humoral immune system is the principal defence mechanism against parasites (Perrigault, Tanguy & Allam 2009).

Cellular immunity seems to play the most important role in protecting against parasite infection (Roch 1999). Haemocytes, the backbone of the bivalve immune system, are potent, multi-functional cells responsible for the main process of the entire cell defence system (phagocytosis), as well as contributing to the transport of nutrients, excretion of pollutants and catabolic products, wound healing, reproduction, shell formation, and secretion of humoral factors (Allam & Raftos 2015; Roch 1999). There are several groups and subgroups of haemocytes (Tiscar & Mosca 2004; Allam & Raftos 2015), with functional variations dependent on specialization in the hosts defences (Hovgaard, Mortensen & Strand 2001, p. 39). Granulocytes seem to be important in primary defences against parasites such as *Marteilia* sp. by phagocytosing sporonts before they can propagate (Butt & Raftos 2008), while hyalinocytes are primarily involved in encapsulation and infiltration but have some degree of phagocytic activity as well (Kuchel et al. 2010). The host haemocyte reaction can differ significantly between mussels infected with parasites and evidence suggests that infections spread the most widely and the most quickly when no host reaction is present (Alderman 1979; Figueras et al. 1991; Villalba et al. 1993). Haemocytes clearly play a

crucial role in defence and resistance against parasites (Tiscar & Mosca 2004; Perrigault, Tanguy & Allam 2009).

1.3 Disease situation in Europe

Pathogens are mainly spread in four different ways: (1) live animal movement, (2) transmission via water, (3) short distance mechanical spread, and (4) long distance mechanical spread (Thrush et al. 2017). Live movement includes the infection of spat in hatcheries that are transported to many different growing sites, potentially leading to transmission to both wild and farmed stocks in the area, and the movement of all age classes, from spat to marketable adults, between suitable grow-out sites (Arzul 2018; Thrush et al. 2017). The movement of molluscs from one area to another is recognised as the main risk factor contributing to the spread of disease (Arzul 2018).

The EEA agreement from 1992 between the EU and EFTA led to the adoption of council directive 2006/88/EC allowing the movement of aquaculture animals from one area to another. This could potentially infect local naïve populations or cause large-scale epizootics across national borders (Arzul 2018). Contrary to fish aquaculture, vaccines are not applicable in farming molluscs due to the lack of an adaptive immunity (Arzul 2018). Control and regulation of mollusc diseases in Europe is therefore entirely based on surveillance and prophylactic measures.

Currently five diseases seem to act as a limiting factor for the total production of molluscs in Europe: marteiliosis, bonamiosis, mikrocytosis, and vibriosis (caused by *Vibrio aestuarianus*) affecting adult populations, and the herpesvirus OsHV-1µvar mainly affecting young oysters (Arzul 2018). All of these are of such importance that they are included in the list of notifiable diseases (see Table 1) set forth by the World Organisation for Animal Health (Office International des Epizooties, OiE), with the exception of OsHV-1µvar that is regulated at the national level in select member states (Arzul 2018). Only one, marteiliosis caused by *Marteilia refringens*, have mussels as a susceptible host (Arzul 2018; Berthe et al. 2004).

Table 1: List of disease and susceptible hosts regulated at the European level (Directive 2006/88/EC) and the national level (Decision 2010/221/EU).

	Diseases	Susceptible host
Exotic diseases	Infection with *Bonamia exitiosa*	*Ostrea angasi, O. chilensis*
	Infection with *Perkinsus marinus*	*Crassostrea gigas, C. virginica*
	Infection with *Mikrocytos mackini*	*C. gigas, C. virginica, O. conchaphila* and *O. edulis*
Non-exotic diseases	Infection with *Bonamia ostreae*	*O. edulis, O. chilensis, O. conchaphila, O. denselamellosa, O. puelchana*
	Infection with *Marteilia refringens*	*O. edulis, O. chilensis, O. angasi, O. puelchana,* ***Mytilus edulis, M. galloprovincialis***
Diseases of importance at the national level	Infection with OsHV-1µvar	*C. gigas*

Source: (Arzul 2018)

Surveillance programs differ between European countries. Active surveillance of diseases monitored at the European level are implemented in a number of countries, including the United Kingdom, Ireland, and Norway, who monitor on the basis of proving free status of these diseases (Arzul 2018). Passive surveillance also occurs, where regions with abnormal mortalities reported by farmers and fishermen are sampled for presumptive diagnosis. However, active surveillance contributes the greatest effort in maintaining control over the spread of disease (Arzul 2018). It is through this type of surveillance that *M. refringens* was first detected in Sweden (2009), South England (2011), Norway (2016), and Northern Ireland (2017) (Arzul 2018; Mortensen et al. 2017a).

Compared to the rest of Europe, Norway's contribution to the shellfish aquaculture industry is minor. Production is limited to a few oyster farms spread across the western coast and roughly 50 mussel farms located mainly in Trøndelag. However, Norway's wild and farmed

stocks are comprised of naïve populations with good health status (Mortensen et al. 2018). The importance of maintaining this status cannot be overstated, as outbreaks of diseases can have potentially massive effects on the populations of molluscs and on ecosystems that depend on them as a food source. Fortunately, importation of molluscs from potentially infected regions is practically non-existent (S. Mortensen, pers. comm), but it is still important to maintain the health status of the naïve stock through surveillance programs and research (S. Mortensen, pers. comm). Even though there seems to be a decline in the wild stocks in certain regions, no reports of massive mortalities have been associated with disease outbreaks (Mortensen et al. 2016, 2017a, 2018).

As part of the surveillance program of mollusc diseases in Norway, the Institute for Marine Research (IMR) perform samplings every six months in accordance with the EU directive 2006/88/EC. The sampling strategy instigated in 2015 defines the sampling period for mussels and oysters to be during the presumed highest prevalence for *B. ostreae* and *M. refringens*. Based on studies in the northernmost areas where *M. refringens* has been detected, this occurs during October (A. Alfjorden, pers. comm).

IMR sample mussels from several points along the Norwegian coast, including Ytre Hvaler in Østfold, Langestrand in Aust-Agder, Hafrsfjord in Rogaland, and Sveio and Bømlo in Hordaland (Figure 4). In 2016, mussels collected from an oyster pool (Agapollen) at Aga, Bømlo were positive for *M. refringens* type M (Mortensen et al. 2017a). Several sampled flat oysters from the same area were also PCR positive for the parasite but showed no histological signs of infection. This prompted an extended survey at IMR under the project *Mussel mortalities* (83737-04) where samples were taken from the area every 3 months in 2017 (Mortensen et al. 2018).

Due to the discovery of marteiliosis at Aga, mussels have been sampled from several other areas in the region, including Håpollen, Espevikpollen, Innerøypollen, Kuleseid and Rogøysund (Figure 4).

1.4 *Marteilia* – an overview

Marteilia spp. are protozoans belonging to the Ascetosporeans, a class of unicellular eukaryotes that exclusively parasitize invertebrates. They are usually found in the marine environment, but have been detected in freshwater and soil, though this is an indolent area of study (Bass, Ward & Burki 2019). The ascetosporeans are obligate intracellular parasites with elaborate spore formation (usually, new groups have been added without this characteristic) and intricate sporulation (Bass, Ward & Burki 2019).

1.4.1 Morphology

The order Paramyxida, comprised of the genera *Marteilia, Paramarteilia* and *Paramyxa,* are characterised by endogenous budding, in which cells mitotically cleave inside mother cells (Bass, Ward & Burki 2019). A fourth genus, *Marteilioides,* seems to be closely related to *Marteilia*, but has not been taxonomically placed yet (Feist et al. 2009).

All species within the three genera *Paramarteilia, Paramyxa,* and *Marteilia* possess a characteristic "cell within a cell" development, where an amoeboid primary cell mitotically cleaves a number of secondary cells within itself (Figure 1). The main diagnostic feature to distinguish the different genera is the nature of the spore: *Paramarteilia* develop one or two bicellular spores from each secondary cell (sporonts), *Marteilia* develop one to six tricellular spores from each secondary cell, and *Paramyxa* develop four tetracellular spores from each secondary cell (Feist et al. 2009). The number of secondary cells, host specification (to some degree), geographical distribution, and infection site are used to differentiate on species level (Feist et al. 2009).

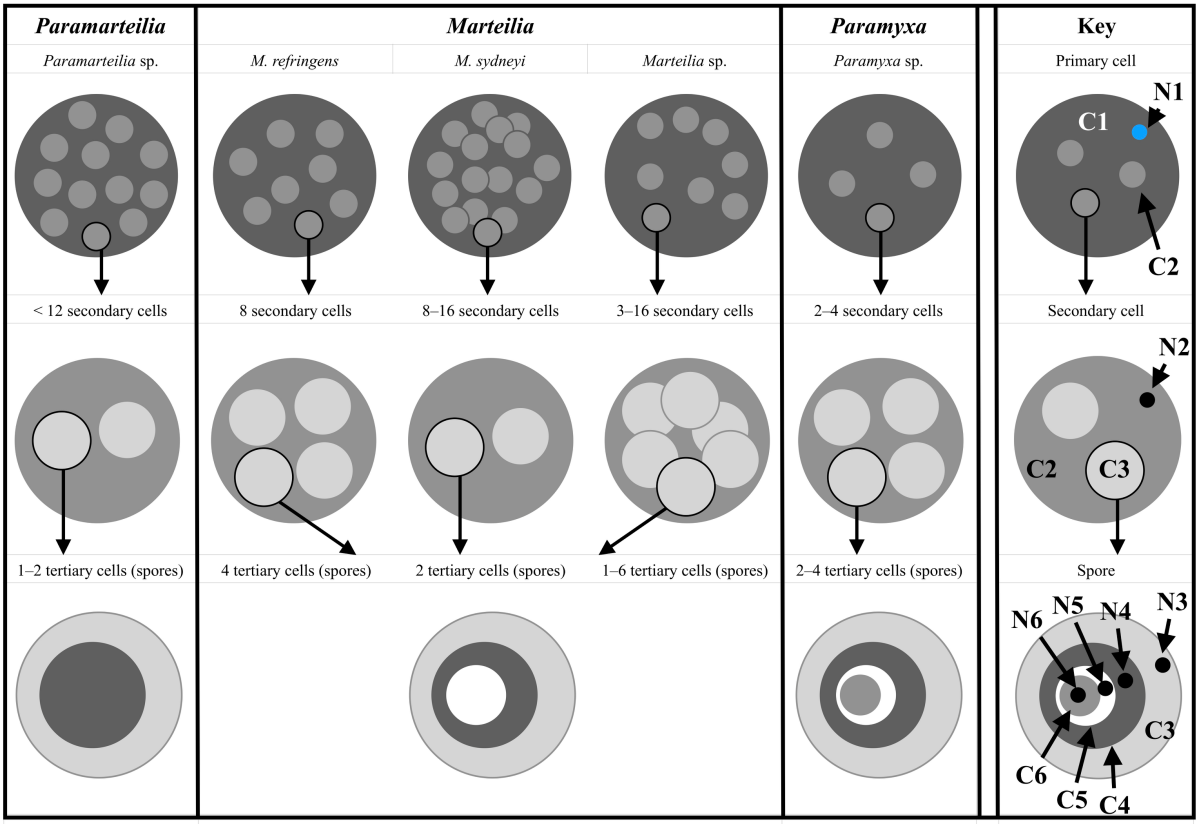

Figure 1: The proposed classification of the phylum Paramyxea based on the nature of spore development. *Paramarteilia* sp. develop up to 12 secondary cells and 1-2 tertiary cells, depending on species. The spore is bicellular. *Marteilia* sp. contain 3-16 secondary cells and 1-6 tertiary cells. Each tertiary cell develops into a tricellular spore. *Paramyxa* sp. develop 2-4 secondary cells and 2-4 tertiary cells. Each tertiary cell develops a tetracellular spore. **Key:** C1: Primary cell/nurse cell; C2: Secondary cell; C3: Tertiary cell/spore; C4-C6: subsequent cells in the tertiary cell, depends on genus; N1-N6: the nucleus of the corresponding cell. Not to scale. Adapted from Feist et al. (2009)

1.4.2 Life cycle

Although the ascetosporeans have been known as impactful pathogens for the last five decades, no complete life cycles have been elucidated (Bass, Ward & Burki 2019). *M. refringens* has been the focus of many life cycle studies as a means to develop effective programmes to control the impact of the disease in endemic zones (Audemard et al. 2001). The complete dynamics between hosts and the environment are still elusive.

The best known segment of the life cycle is the development of *M. refringens* in flat oysters, *O. edulis* (Audemard et al. 2001). The infection is seasonal and partially dependent on temperature (Berthe et al. 2004). The primary stages appear at the onset of spring (May-June), coinciding with an increasing in water temperature (Berthe et al. 1998). The primary stages usually occur in the stomach epithelium, but has also been found in the gills, labial palps, and the connective tissue of the mantle in *M. edulis* (Garcia et al. 2009). The

development from the primary stage to mature spores is complex, involving several endogenous buddings. This process is shown in Figure 2.

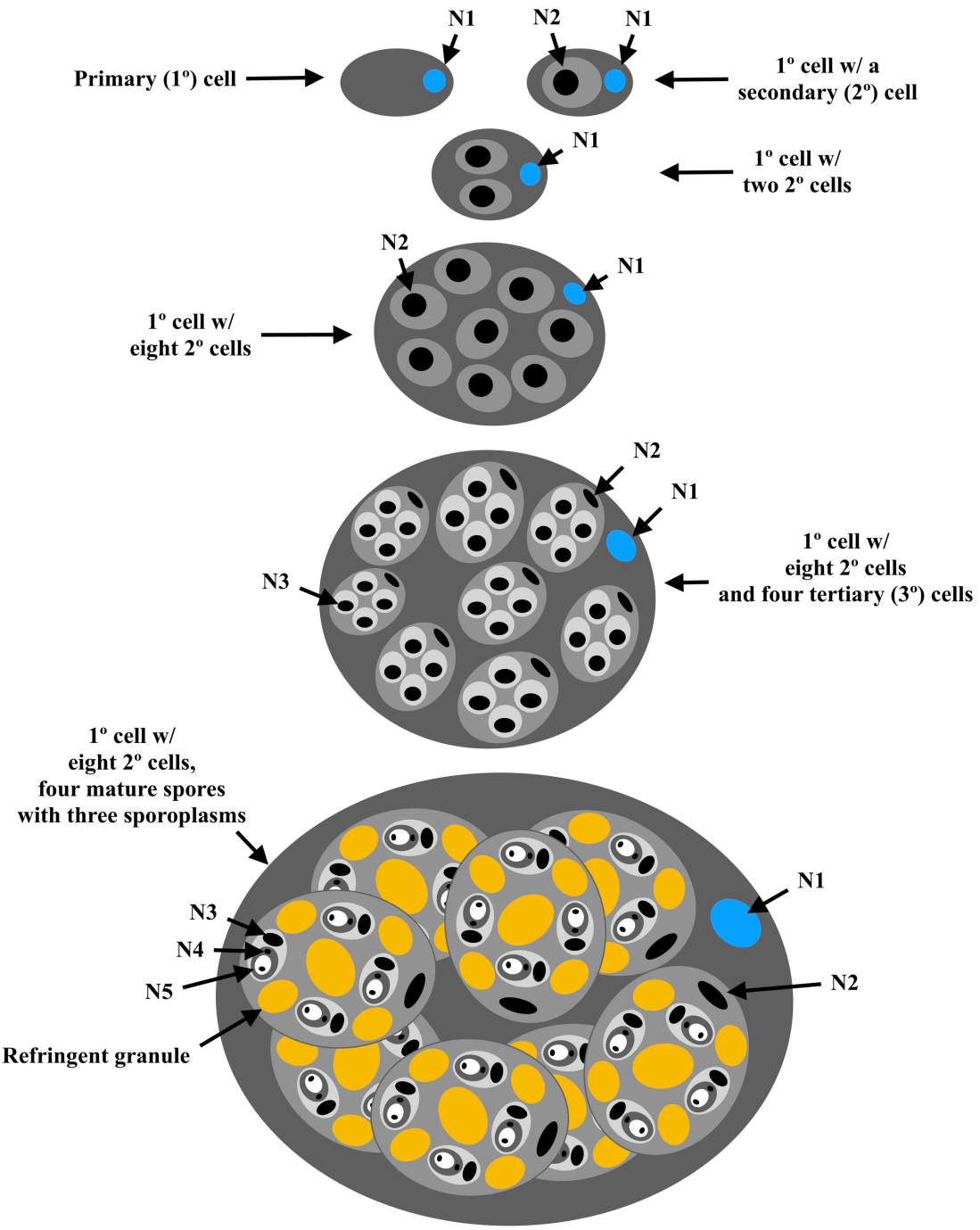

Figure 2: The 'simplified' development of *Marteilia refringens* in the flat oyster *Ostrea edulis*. N1: nucleus of primary cell; N2: nucleus of secondary cell; N3: nucleus of tertiary cell/spore; N4: nucleus of the middle sporoplasm cell; N5: nucleus of the final sporoplasm cell. The first step of the infection involves the penetration of a primary (1°) cell either in the stomach epithelium or digestive tubules. The 1° cell either contains a secondary (2°) cell prior to, or develops it immediately following, the infection (Carrasco et al. 2015). The 2° cell cleaves to form two 2° cells, followed by cleavage into eight 2° cells. Each 2° cell endogenously cleaves into four tertiary (3°) cells, each containing a nucleus (N3). These tertiary cells develop two other cells, one within the other, forming a tricellular spore (three sporoplasms). Each 2° cell also develops several refringent bodies (yellow circles). Adapted from Feist et al. (2009), Carrasco et al. (2015), and Berthe et al. (2004).

The primary stage contains a single primary cell and a secondary cell within, which eventually cleaves to form up to eight secondary cells called presporangia (Carrasco, Green & Itoh 2015).

It has been proposed that the presporangia-stage of the parasite migrate to the ducts and digestive tubules of the bivalve host. The process of this migration is not understood (Carrasco, Green & Itoh 2015). It is within the digestive tubules that the bulk of maturation occurs (Carrasco, Green & Itoh 2015; Villalba et al. 1993). The early stages of sporulation (ES) consists of 'pseudoplasmodia'; the primary cell containing only sporangia (Villalba et al. 1993). Sporangia develop four spore primordia (number dependent on *Marteilia* species) within the pseudoplasmodia, which is considered the intermediate stage of sporulation (IS) (Villalba et al. 1993). The final developmental stage is the advanced stage of sporulation (AS), where the spore primordia have matured into sporonts with refringent granules (Villalba et al. 1993).

The final stage of the *Marteilia* infection is the release of spores into the lumina of the digestive tubules (Carrasco, Green & Itoh 2015; Bass, Ward & Burki 2019). The process causes the disruption and physiological dysfunctions of the digestive tubules, usually with fatal result (Carrasco, Green & Itoh 2015). Sporulation usually occurs in the autumn months, coinciding with the highest prevalence of the parasite (Carrasco, Green & Itoh 2015). However, some studies suggest that no such temporal pattern with a marked seasonality occurs in mussels, where sporulation has been shown to occur year round (Villalba et al. 1993).

What occurs after sporulation is not fully known. Transmission of the parasite to naïve oysters in oyster ponds, so called 'claires,' without any residual infected oysters has been successful (Berthe et al. 1998). This suggests that infective stages can be present in environments that have previously had outbreaks of the disease, indicating presence in some other host or a reservoir where spores can survive (Berthe et al. 1998). A complex life cycle with one or more intermediate hosts has been postulated by many authors, but conclusive evidence has not been presented (Audemard et al. 2001).

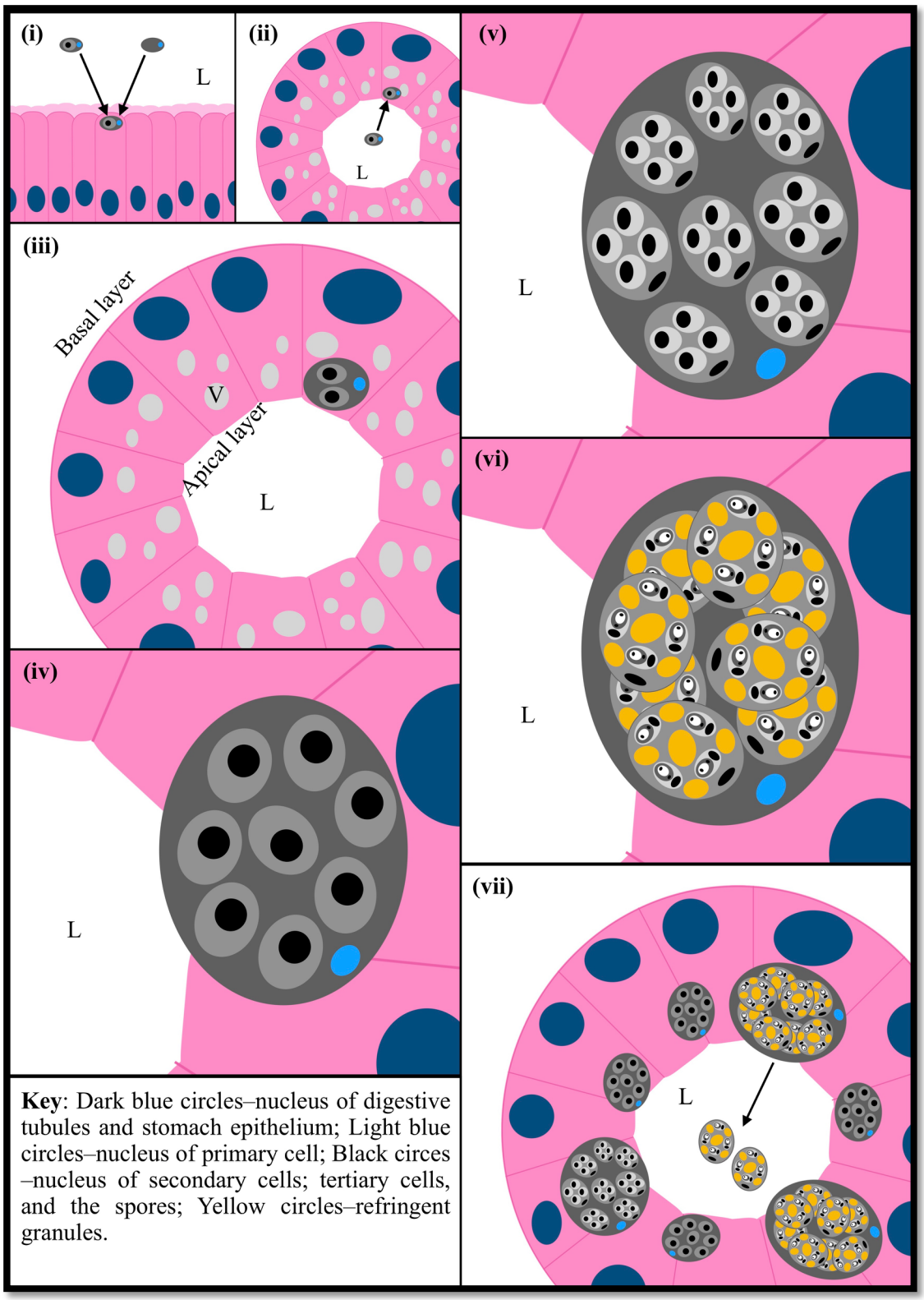

Figure 3: The schematic overview of infection of *Marteilia* sp. in a bivalve host. Ref. *Figure 2* for labels for each developmental stage. Ref. **key** for colour-coding of the host tissue anatomy. **(i)** Penetration of primary cell into the stomach epithelium or **(ii)** penetration of primary cell in the digestive tubules. **(iii)** the primary cell develops two secondary cells in the apical layer of the digestive tubule (towards lumen, L). V: vesicle containing digestive enzymes. **(iv)** the primary cell develops eight secondary cells. This stage corresponds to the early stage (ES). **(v)** Each secondary cell develops four tertiary cells. This stage corresponds to the intermediate stage (IS). **(vi)** Each tertiary cell has developed into a tricellular spore. Refringent granules can be seen. This stage corresponds to the advanced stage (AS). **(vii)** Sporulation occurs from AS and large numbers of sporonts (secondary cells with tricellular spores) can be seen in the lumen. Different stages are usually present concurrently in heavily infected hosts.

1.4.3 Taxonomy

The genus *Marteilia* was first proposed by Grizel et al. (1974) when the type species *M. refringens* was described as the causative agent for "digestive gland disease" in the flat oyster *Ostrea edulis* (Comps 1970). Since then, several species have been described parasitizing different bivalves (Table 2) as well as other invertebrates, such as the European edible crab, *Cancer pagurus* (Feist et al. 2009). Since 2013, at least four new species of *Marteilia* have been described: *Marteilia cochillia* (Carrasco et al. 2013), *Marteilia octospora* (Ruiz et al. 2016), *Marteilia tapetis* (Kang et al. 2019), and the focus of this thesis, *Marteilia pararefringens* (Kerr et al. 2018).

Table 2: Marteilia and *Marteilioides* species and their respective hosts. The original citation and distribution are also included. A=Atlantic; M=Mediterranean; P=Pacific; I=Indian ocean

Parasite	Host		Original citation	Origin (Ocean)
M. refringens	*Ostrea edulis*	European flat oyster	Grizel et al. (1974)	Europe (A/M)
	Ostrea stentina	Flat oyster	Elgharsalli et al. (2013)	Tunisia (M)
	Xenostrobus securis	Mussel	Pasqual et al. (2010)	Spain (A)
	Chamalea gallina	Venus clam	López-Flores et al. (2008)	Spain (M)
M. maurini	*Mytilus edulis* and *Mytilus galloprovincialis*	Blue mussel Mediterranean mussel	Comps et al. (1982)	Europe (A/M)
M. pararefringens	*Mytilus edulis*	Blue mussel	Kerr et al. (2018)	Europe (A)
M. sydneyi	*Saccostrea glomerata*	Sydney rock oyster	Perkins and Wolf (1976)	Australia (P/I)
M. lenghei	*Saccostrea cucullata*	Natal rock oyster	Comps et al. (1986)	Persian Gulf (I) & Australia (I)
M. octospora	*Solen marginatus*	Grooved razor clam	Ruiz et al. (2016)	Spain (A)
M. tapetis	*Ruditapes philippinarum*	Manila clam	Kang et al. (2019)	Japan (P)
M. cochilla	*Cerastoderma edule*	Common cockle	Carrasco et al. (2013)	Spain (M/A)
M. christienseni	*Scrobicularia piperata*	Clam	Comps (1983)	France (A)
Marteilia sp.	*Argopecten gibbus*	Calico scallop	Moyer et al. (1993)	Florida (A)
Marteilioides chungmuensis	*Crassostrea gigas*	Pacific oyster	Comps et al. (1986)	Korea (P)
	Crassostrea nippona	Iwagaki oyster	Itoh et al. (2014)	Japan (P)
	Crassostrea ariekensis	Sumione oyster	Limpanont et al. (2013)	Korea (P)

Taxonomy of *Marteilia* species is constantly under review, with species being synonymized and novel species being discovered fairly regularly. Two particular species that have been scrupulously discussed are *M. refringens* and *Marteilia maurini*. *M. refringens* was first

described in 1974 in populations of flat oysters, *O. edulis,* in France (Grizel et al. 1974). *M. maurini* was first described in 1982 parasitizing the mussel *M. galloprovincialis* imported to France from Venice Lagoon in Italy and later in 1983, parasitizing *M. edulis* from France (Comps et al. 1982; Auffret & Poder, 1983; Zrnčić et al. 2001). The two *Marteilia* species were distinguished based on ultrastructural characteristics and host specificity (Grizel et al. 1974; Perkins 1976; Comps et al. 1982; Figueras & Montes 1988).

The ultrastructural characteristics were based on haplosporosome measurement, spore wall thickness (concentric membranes), and higher level structural criteria, such as number of secondary and tertiary cells present (Longshaw et al. 2001). Haplosporosomes have been deemed highly pleomorphic and therefore not suitable to distinguish on species level. Spore wall thickness depends on maturity stage and is only of value if measured on a mature spore, which is difficult to deduce. Higher level structure is identical between the two species, i.e. same number of secondary and tertiary cells (Longshaw et al. 2001). Differentiating species on the basis of ultrastructural criteria is also a controversial area as parasites can display different morphology depending on host (Zrnčić et al. 2001).

M. maurini was suggested to exclusively parasitize the Mediterranean mussel, *M. galloprovincialis*, while *M refringens* seemed to only infect the European flat oyster, *O. edulis* (Grizel et al. 1974; Comps et al. 1982). However, host specificity was not a viable criterion as *M. refringens* was found infecting the mussel *M. galloprovincialis* (Villalba et al. 1993; Robledo & Figueras 1995). As Longshaw et al. (2001) has discussed, the use of ultrastructural criteria is unreliable in differentiating on species level, and host specificity has been challenged by Villalba et al. (1993) and Robledo & Figueras (1995). Although existing criteria were not sufficient in differentiating these two species, this had not yet been confirmed by complete DNA sequence data (Longshaw et al. 2001).

In parasitological molecular taxonomy, small subunit ribosomal RNA (SSU rRNA) is generally used to differentiate species due to the availability of sequences and the variable regions intercalated with conserved sequences among organisms (Hillis & Dixon, 1991; Page & Holmes, 1998; Balseiro et al. 2007). 18s rDNA sequence was evaluated in both *M. refringens* and *M. maurini* in 1999 and 2000, with no distinguishable differences found between them (Le Roux et al. 1999; Berthe et al. 2000) Therefore this criterion is not particularly useful in discriminating *Marteilia* sp. (Balseiro et al. 2007). Using polymerase

chain reaction restriction fragment length polymorphism (PCR-RFLP) on the more quickly-evolving internal transcribed spacer (ITS) 1 rDNA region, it was found that 40 positions on this gene was polymorphic between *M. refringens* and *M. maurini* (Le Roux et al. 2001; Kerr et al. 2018). Le Roux et al. (2001) suggested that this distinction matched host specificity almost perfectly, but this was refuted by López-Flores et al. (2004) and Novoa et al. (2005) when both species were found in both hosts. López-Flores et al. (2004) attempted to differentiate the *Marteilia* species by analysing an intergenic spacer (IGS) region (358 bp), but this was also unsuccessful.

The arguments for preserving *M. maurini* as a species were fading and eventually suggested to be regarded as synonymous with *M. refringens* (Le Roux et al. 2001; Balseiro et al. 2007). The suggestion was accepted, and the two species were now considered conspecific, with *M. refringens* M-type and O-type to differentiate them, respectively (EFSA, 2007). Synonymizing was not a trivial decision as any new discoveries of either type would have to be reported as *M. refringens*, notifiable by OiE and the European Commission (Directive EC/2006/88) (Kerr et al. 2018). Several sites in Northern Europe previously negative for *M. refringens* have consequently been declared positive for the disease.

M. refringens type M was found for the first time in Norway in 2016 from mussels sampled Aga, Bømlo. These mussels were cohabiting with flat oysters that did not become infected. No verified reports of *M. refringens* type O have been registered here (or any other areas in Northern Europe) and no abnormal mortalities have been attributed to the parasite in neither mussels nor oysters (Kerr et al. 2018). Due to the lack of *M. refringens* type O detected in these regions, despite cohabitation, Kerr et al. (2018) began extensive sequencing of the full-length ribosomal RNA gene arrays of all samples of *M. refringens* and compared them to a comprehensive literature survey. The purpose of this study was to assess whether it was possible to distinguish the two profiles based on gene sequence alone. Kerr et al. (2018) gathered samples from *M. refringens*-infected mussels from France, Norway, Spain, Sweden, and the UK and verified that the mussels were exclusively *M. edulis* (not *M. galloprovincialis* or a hybrid between the two), based on method described by Inoue et al. (1995) and tested by Bignell et al. (2008). They performed a meta-analysis of all available ITS-1 and IGS sequences from *Marteilia* sp. and recognised five signatures that could distinguish *M. refringens* M- and O-type. This differs from early studies of ITS-1 and IGS regions as these

have been based on short amplicons generated by weakly resolved primers instead of whole sequence analysis.

The authors have therefore suggested that grouping *M. refringens* O-type and M-type should be discontinued. Instead, they argued that *M. refringens* should be used for the species infecting oysters (previously O-type) and *Marteilia pararefringens* sp. nov. for the parasite infecting mussels (previously M-type, initially *M. maurini*), with different geographic distributions. They claim that this is sufficient evidence to segregate these into separate species with different hosts and life cycles (Kerr et al. 2018).

1.5 Objectives

Agapollen represents the northern-most distribution of any *Marteilia* sp. in Europe. The semi-closed system of the oyster pond represents a unique opportunity to study the life cycle and parasite progression of *M. pararefringens* in *M. edulis*. The objective of this thesis can be divided into two parts:

1. A description of the transmission and progression of *M. pararefringens* infection in *M. edulis* through a cohabitation experiment at Aga, Bømlo for the duration of one year.
2. Examine the occurrence of *M. pararefringens* in fauna samples from the environment of the study population.
3. Perform cohabitant transmission trials in a controlled environment (laboratory) to see whether the parasite can infect healthy mussels without an intermediate host.

Both PCR and histological analyses were used in the cohabitant studies (field & laboratory), while only PCR screening was performed on fauna samples.

2 Material and Methods

2.1 Study Site

The oyster pond ‘Agapollen’ (Figure 4) is located in Bømlo municipality, Hordaland (59°50'23.6"N 5°14'49.7"E). It contains two conduits for water transmission: a connection to a freshwater pond and a channel where saltwater circulates. The channel is connected to Håpollen, which leads to Bømlafjorden. The pond is around 150 x 200 m wide and 6 m deep, with soft sediments (S. Mortensen, pers. comm).

The site was previously used to rear European flat oysters, *O. edulis*, until production ceased in 2014. Screening for marteiliosis has been ongoing since 2006, but only flat oysters were sampled until the introduction of mussels into the surveillance program in 2016. This coincided with the first positive results of marteiliosis in this area. The dynamics of *M. pararefringens* sp. nov. in mussels and the surrounding fauna was only briefly studied in 2017, with tri-monthly samplings from mussels in the channel. These could have been infected multiple times, which makes the establishment of a hypothesis on transmission time difficult. Naïve mussels were deployed at the site to study the case further.

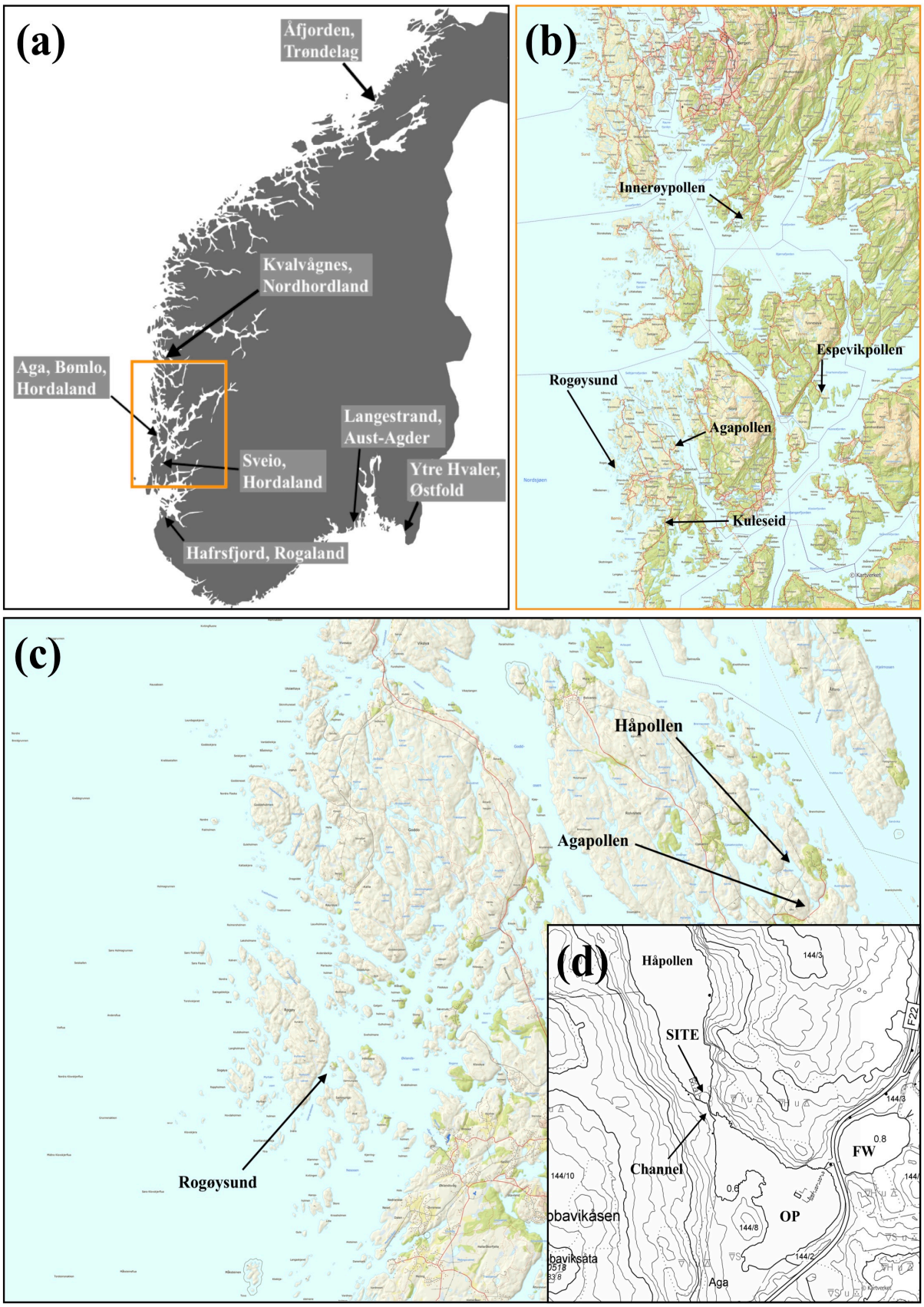

Figure 4: Location of the experimental cultures at Aga, Bømlo. (a) Sampling sites as part of the surveillance program performed by the Institute for Marine Research in Bergen. (b) 1:300000 The surveillance areas that were included after the discovery of *M. pararefringens* sp. nov. at Aga. (c) 1:50000 280 healthy mussels were gathered from Rogøysund and placed in oyster baskets in Agapollen. (d) 1:2500 Detailed view of Agapollen, OP; FW: freshwater source; SITE: deployed mussels in rearing cages; Channel: connecting the oyster pond with Håpollen, it's saltwater source. Sampling from site occurred every six weeks, from May 2018 to April 2019. About 30 mussels were sampled from the rearing cages deployed at the start of the study. Fauna and plankton samples were also taken in the area surrounding the channel, as well as from the raft located in the middle of the pool. Kartutskrift.no

2.2 Mussels

About 660 healthy mussels (length = 58 mm ± 12 mm; weight = 24 g ± 17 g), *Mytilus edulis*, were collected at Rogøysund: 280 in May for the first deployment, 280 in October for the second deployment and 100 for the transmission trial in the laboratory at IMR. To verify the genotype of the mussels, a genetic analysis was performed at CEFAS (D. Bass, pers. comm; Kerr et al. 2018).

Before placement, 30 mussels were screened for each group to verify that they were negative for *Marteilia* sp. (null-sample). The same null-sample was used for both the second deployment and cohabitation trial in the laboratory as these were started concurrently.

2.2.1 Cohabitation trial in the field

Mussels were transported in Styrofoam boxes to the sample site at Aga where they were deployed in SEAPA Ltd 15 L/20 mm mesh oyster baskets (Figure 5) and placed at the sampling site (Figure 4) in the subtidal zone. They were secured in place using ropes and cable ties to a pipe running parallel to the channel. To make sure the cages would not move rocks were placed on top.

Figure 5: The setup of the oyster baskets (SEAPA Ltd 15L/20 mm mesh) used to house the mussels during the cohabitation experiment. 250 mussels were distributed between two such baskets and kept in a channel connecting the oyster pond at Aga, Bømlo with Håpollen to the north. The baskets were placed in the subtidal zone, tied to a pipe that ran from Agapollen to Håpollen.

The 250 mussels per deployment were divided in two oyster baskets (about 125 each). The secondary, parallel study in October was used to study whether transmission of the parasite could occur during sporulation in autumn. October was chosen based on data from 2017 which showed sporulation stages in mussels from Agapollen during this period. The potential infection of these mussels could give further insight into the transmission period.

2.2.2 Cohabitation trial in the laboratory

To demonstrate whether *M. pararefringens* can be transmitted between mussels without the presence of an intermediate host, a cohabitant study in the laboratories at IMR Bergen was established. 50 infected mussels from Agapollen, divided into two baskets, were suspended

above a bed of 100 healthy mussels from Rogøysund (Figure 6). To mimic natural water flow, water flow was turned on at 08:00 and turned off at 15:30. An aquarium circulation pump was used to circulate the water when the flow was turned off.

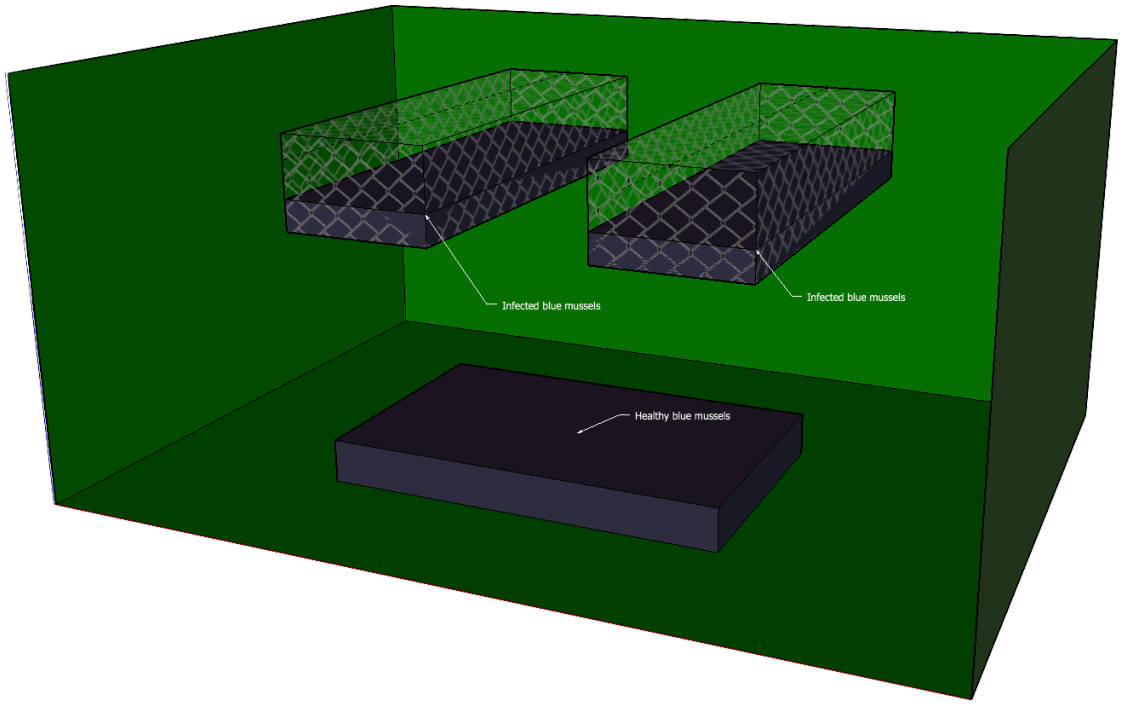

Figure 6: Schematic set-up for the closed cohabitant study used to verify whether *Marteilia pararefringens* sp. nov. can infected healthy oysters without an intermediate host. 50 infected mussels collected inside the oyster pond at Aga, Bømlo were placed in two baskets floating above a bed of 100 non-infected mussels from Rogøysund. In order to mimic the tides, water circulation was turned on and off corresponding to the work day (on from 8-15:30, off until the following day). The experiment lasted two months.

2.3 Sampling protocol

2.3.1 Cohabitation trial in the field

In order to illustrate the life cycle of *M. pararefringens*, samples were taken from the oyster baskets at Aga every six weeks from May 2018 to March 2019 (*Table 3*). The sample size was 30 mussels of no specific size, 15 from each basket. The mussels were deposited into a Styrofoam box with cooling elements and damp cloths for transport to IMR. This protocol has proven sufficient in keeping the animals alive for extended periods of time.

Table 3: Sampling from the oyster cages at Aga. Histology screening and PCR sample size corresponds to the same individuals, but some slides could not be used for screening as they did not contain digestive tubules.

Date	Sample size deployment 1		Sample size deployment 2	
	Histology screening	PCR	Histology screening	PCR
28.05.2018	30	30	-	-
04.07.2018	30	30	-	-
29.08.2018	29	29	-	-
08.10.2018	29	29	-	-
09.10.2018	-	-	-	30
19.11.2018	23	24	30	30
03.01.2019	-	-	30	30
11.02.2019	-	-	30	30
25.03.2019	-	-	30	30
Total infected/positive	71	76	0	0
Total	141	142	120	150

2.3.2 Cohabitation trial in the laboratory

The transmission trial at IMR lasted for two months, from October to December 2018. At the end of the trial, 30 of the infected mussels were removed and sampled for histology and PCR analysis. The tank was emptied, scrubbed, and refilled with clean water. This procedure was completed three times (once every two days) before 30 of the presumable healthy mussels were removed and sampled for histology and PCR analysis. The purpose of this step was to remove any potential spores circulating in the water to minimize the risk of false positives.

2.3.3 Fauna samples

Fauna samples were collected in conjunction with sampling of mussels at Aga. All fauna samples were PCR analysed only. Shrimp were sampled from the channel and from the oyster pond using nets. The digestive gland was extracted on site using sterile scalpels and placed in ethanol. Shrimp were not discriminated based on size. Bristleworms were collected from oyster growth racks in the oyster pond. Each individual was cut into small pieces (approximately 2-3 mm in length) and placed in ethanol. They were not discriminated based

on size. Plankton samples were gathered using a WP2 180 µm mesh plankton net, submerged around 2 meters below the surface and dragged behind a boat in the oyster pond. Plankton samples were placed in ethanol.

2.4 Processing of material

2.4.1 Histology

IMR's protocol for processing samples for histology was followed (Appendix A). Below is a summary of this method.

After measuring length and mass of each individual, the mussels were opened and cut dorso-ventrally across the anterior end of the organism, towards the umbo, as illustrated in Figure 7. In this particular study, only the organs involved in digestion were seen as relevant as those are the primary target tissues for most *Marteilia* sp.

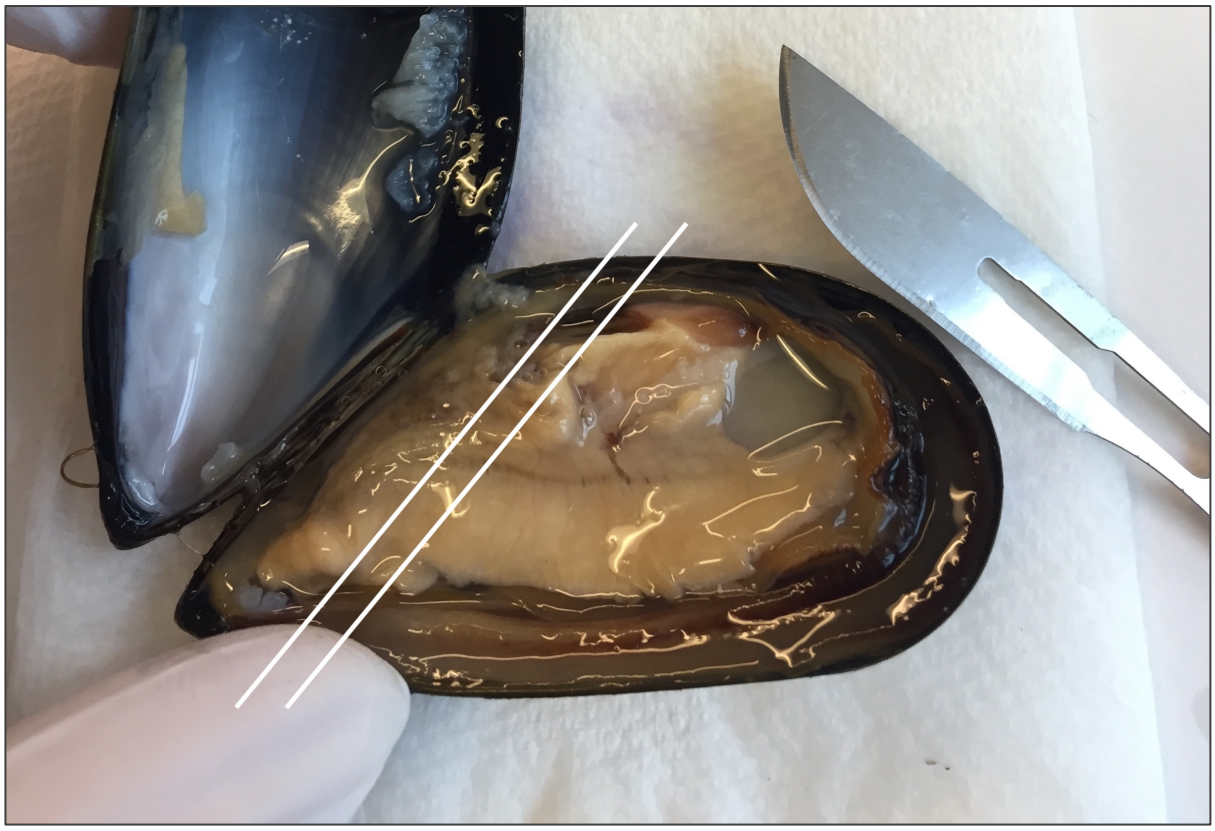

Figure 7: Dissection of mussels was performed in the laboratory at IMR. Mussels were dorso-ventrally cut across the anterior end of the organism (diagonal lines), towards the umbo (left). A section containing all organs was removed and placed in cassettes and submerged in Davidson's fixative. Two samples for PCR analysis were also removed. Remaining tissue was submerged in ethanol and stored in a freezer.

The dissected tissue was placed immediately into cassettes marked with the species, sample number and location, and added to Davidson's fixative solution with added acetic acid and left for a minimum of 48 hours to a maximum of seven days.

For dehydration, infiltration, embedding, sectioning, and staining, a standard histology protocol was followed (Appendix A) based on Howard et al. (2004). Two 3µm sections were cut from each mussel with ~200 µm between the sections. Haematoxylin, Eosin and Saffron (HES) staining was applied.

2.4.2 PCR

All mussels and fauna samples were PCR analysed for presence of *Marteilia*. All mussel samples were run at IMR, while fauna samples were sent to the Centre for Environment, Fisheries and Aquaculture Science (CEFAS) in Weymouth, England for analysis. Standard protocol was followed (Appendix B). Summary given below.

DNA Isolation

During dissection of mussels for histology, small (ideally ca. 0.25-1 mm^3) samples of the digestive tissue from each individual was excised and transferred to a 2 ml Eppendorf tube containing 1.5 ml ethanol. If the samples were not to be processed within a short time after sampling, they were stored at -20°C. QIAGEN QIAamp DNA Mini Kit REF51306 was used in the isolation of DNA from mussel tissue (Appendix B). The DNA was eluted in 50 µl distilled water. The concentration was analysed using a NanoDrop ND-1000 Spectrophotometer. The samples were diluted to a concentration of 100 ng/µl. Samples under 100 ng/µl were not diluted.

Polymerase Chain Reaction

The procedure was performed in 25 µl reactions consisting of 1X PCR buffer, 1X Q-solution, 0.5 µM of both the forward primer (M2A 5'-CCGCACACGTTCTTCACTCC-3'; Pr4) and reverse primer (M3AS 5'-CTCGCGAGTTTCGACAGACG-3'; Pr5) (Le Roux et al. 2001), 0.2 mM of dNTPs, 0.6 U HotStarTaq DNA polymerase and 100 ng/µl DNA.

23 µl of the mixture was added to individual wells on a PCR tray and a plastic film was put on top for transport between labs. 2 µl of DNA sample was added to each respective well and

two positive (M-type and O-type) and negative controls included. The tray was centrifuged and put into a PCR machine (ProFlex PCR System) for DNA amplification.

Amplification

The machine was set to hold at 95°C for 15 minutes. 35 cycles of denaturation at 94°C for one minute, annealing at 60°C for one minute, extend at 72°C, and extend/hold at 72°C for 10 minutes. The tray was incubated at 4°C after amplification.

Agarose gel electrophoresis

1% agarose gel (LONZA SeaKem® LE Agarose) in 1x TAE buffer with added GelRed Nucleic Acid Stain (Biotium art. Nr41003) was made. 8 µl of amplified DNA products was mixed with 2 µl of 5X Green GoTaq® loading buffer. This was then placed into wells in an agarose gel and run at 60 V for approximately 2 hours before it was placed an iBright CL1000 scanner where digital images were taken. Positive results were shown as light bands of correct length.

2.4.3 Restriction Fragment Length Polymorphism

In order to identify the *Marteilia* species (*Marteilia refringens* sensu lato) present in the positive samples from PCR analysis, the method of restriction fragment length polymorphism (RFLP) was used on a subset of the samples. The enzyme *Hha* I cleaves the amplified partial 18S rRDNA in specific regions that can be analysed using gel electrophoresis. *M. pararefringens* (*M. refringens* type M) positive will have four light bands at 157 bl, 156 bp, 68 bp, and 31 bp, while *M. refringens* (type O) will have three light bands at 226 bp, 156 bp, and 31 bp.

20 µl of total RFLP mix was prepared: 1X Buffer C, 0.1 µg/µl BSA, and 0.5 U/µl *Hha* I enzyme was mixed with RNAase free and 10 µl PCR product of each sample. The mixture and samples, including M- and O type positive controls and one negative control, were added to microtubes, placed in a PCR machine and incubated at 37°C for 1 hour. A 2% agarose gel with 1x TAE buffer was prepared. Loading buffer was added directly to each microtube and 18 µl was placed in wells on the gel. Ladders were placed in wells on each flank of the samples. The gel was run at 60 V for 2-3 hours. iBright CL1000 was used to take digital images.

2.5 Screening

Slides were scanned using Hamamatsu NanoZoomer S60 and screened using NDP.view2 software. All screening was performed on 24-inch monitors, HP ZR2440w and Samsung SyncMaster 2493HM, at 400x magnification. These have approximately the same field of view as the LEICA DMRBF microscope at the same magnification.

2.5.1 Scoring

The method of scoring the mussels for intensity of infection was based on Villalba et al. (1993). Every mussel was rated according to degree of proliferation of *Marteilia*-cells present in the digestive system. The degree was based on a scale from 0 to 5 (Table 4).

Table 4: Scoring system for mussels infected with *Marteilia* sp.

Intensity		Description
Uninfected	0	Parasite not detected
Light infection	1	Parasite confined to stomach epithelium. Early infection.
Moderate infection	2	The percentage of infected digestive tubules (PIDT) is less than 10%
Heavy infection	3	10% < PIDT < 50%
	4	50% < PIDT < 90%
	5	PIDT > 90%

Only entire digestive tubules (in the field of view) and with a visible lumen were counted. *Marteilia*-cells located in the lumina were not counted since these cannot with certainty be attributed to any proximal branch. The percentage of infected digestive tubules (PIDT) was used as the definition for moderate and heavy infections. PIDT was calculated for every mussel gathered from the culture cages at Aga, as well as from the transmission trial in the laboratory at IMR. This was done by random selection of 10 sites at 400x magnification and counting the non-infected (NDT) and infected digestive tubules (IDT). The following formula was used to calculate the PIDT:

Equation 1: The percentage of infected digestive tubules.

$$\mathrm{PIDT} = \frac{\mathrm{IDT}}{\mathrm{IDT+NDT}} * 100$$

For consistency, only the digestive tubules entirely visible within the field of view (i.e. not outside the screen) and with a visible lumen were counted. *Marteilia*-cells located in the lumen were excluded as these cannot be attributed to the specific digestive tubules present in the field of view.

The relative abundance of three different stages was estimated for moderate and heavy infections. The stages were categorized based on when in the sporulation phase the cells were: Early stages of sporulation (ES) – pseudoplasmodia with only secondary cells; Intermediate stages of sporulation (IS) – pseudoplasmodia with secondary cells and spore primordia; Advanced stages of sporulation (AS) – pseudoplasmodia with secondary cells and refringent spores. The percentage of each stage (PES, PIS, and PAS) was calculated using the following formula:

Equation 2: Percentage of each stage of sporulation of Marteilia sp.

$$\mathrm{PXS} = \frac{\mathrm{XS}}{\mathrm{ES+IS+AS}} * 100$$

Where "X" represents early, intermediate, or advanced stage.

The mean number of parasites per infected digestive tubule (MNPIDT) was calculated using the following equation:

Equation 3: Mean number of parasites per infected digestive tubule.

$$\mathrm{MNPIDT} = \frac{\mathrm{ES} + \mathrm{IS} + \mathrm{AS}}{\mathrm{IDT}}$$

Each sampled mussel was also scored based on the degree of host haemocytic reaction. An arbitrary scale was used, ranging from 0 (no infiltration of haemocytes in the digestive gland) to 3 (heavy infiltration of haemocytes in the digestive gland). 1 and 2 were intermediate values (light and moderate, respectively).

2.5.2 Statistical analysis

Statistical analyses were done using Statistica (13.3) software (StatSoft). Temporal changes in infection intensity (not intensity score, i.e. '0' values removed) was examined using analysis of variance (ANOVA). Analysis of covariance (ANCOVA) was used when examining the relationship between intensity score and host haemocytic reaction. Levene's test for homogeneity of variances was used to verify the validity of the tests.

Correlations between PIDT and MNPIDT, PES, PIS, and PAS was examined using Spearman rank correlation coefficients. Double-zeros (i.e. non-infected individuals) were excluded in order to avoid spurious correlations (Ludwig & Reynolds 1988, p.155).

Fisher Exact Tests (FET) were used to examine binomial difference between two samples, such as differences in prevalence and proportion of infected digestive tubules (IDT).

3 Results

3.1 Cohabitation trial in the field

A total of 292 mussels were included in the cohabitation study in the field, from May 2018 to March 2019. All mussels were sampled for PCR and histological analysis, with the exception of one individual from the October-sample that was too moribund for histological examination and the null-sample for the second deployment, which were only PCR analysed.

3.1.1 Progression of the infection in the host

The different stages of *M. pararefringens* were found in histological sections of the mussel *M. edulis* (Figure 8 & Figure 9). Early stages (ES) were located in the epithelium of the stomach, ducts, and digestive tubules. Primary cells of ES contained one to eight secondary cells, and consequently varied in size. No length measurements were taken of the stages. Intermediate stages (IS) were found only in the digestive tubules. IS had darker secondary cells, with what appeared to be a white boundary surrounding each. The secondary cells seemed to cluster in groups. Advanced stages (AS) were found only in the digestive tubules. AS contained refringent granules that were grey or green in colouration (HES staining).

Vegetative stages were present only in the stomach epithelium in some individuals, indicating the earliest stages of the disease. The disease spread further to the ducts and digestive tubules. Only early stages were seen in the stomach epithelium ducts, but all stages were present in the digestive tubules (Figure 8). Sporulation only occurred in the digestive tubules. Sporonts enclosing the spores were seen in large numbers in the lumina of some individuals (Figure 9e-f).

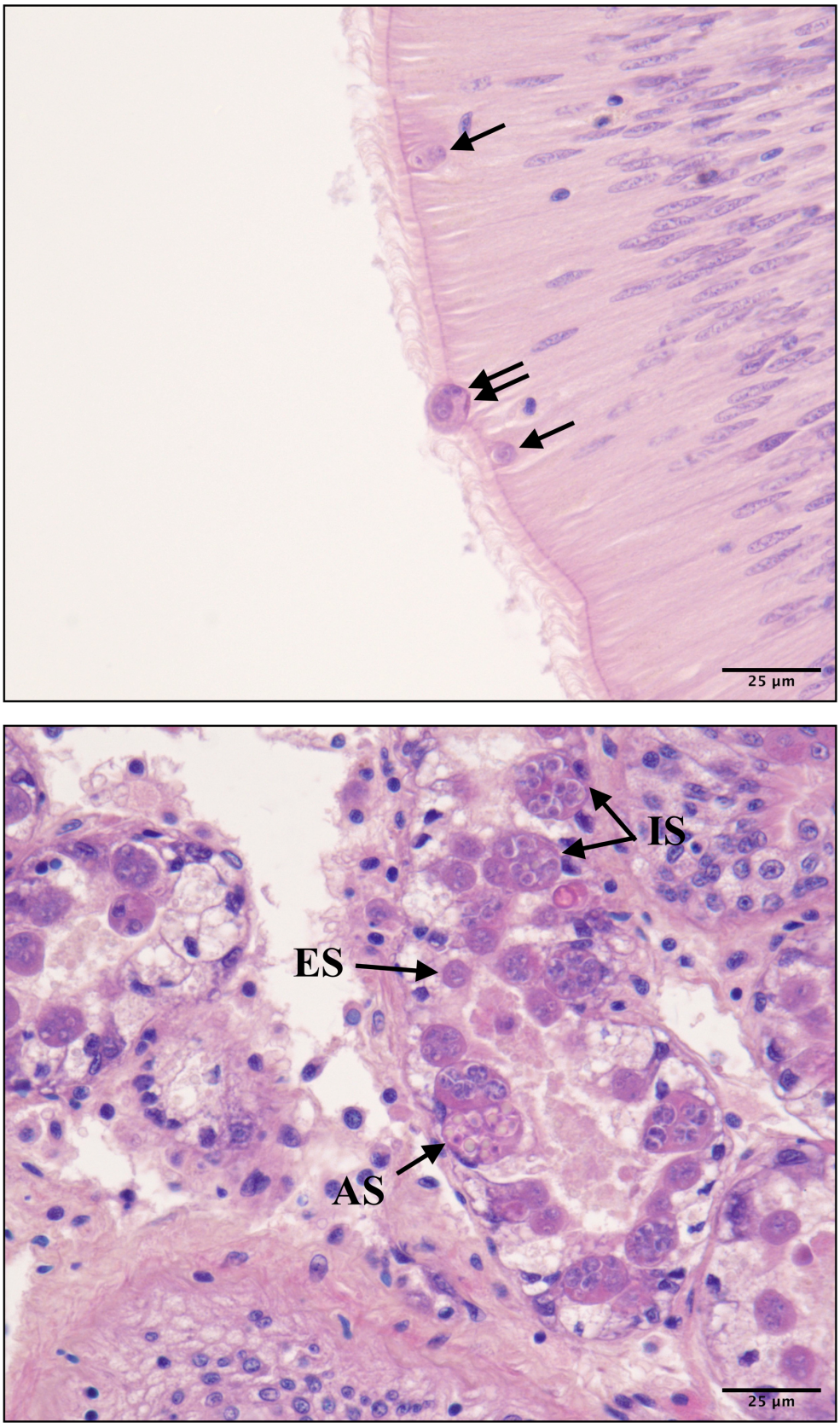

Figure 8: Marteilia pararefringens stages infecting *Mytilus edulis*. HES staining. **Top:** Three early stages (ES; arrows) of the parasite present in the stomach epithelium. One parasite (double arrows) is either penetrating into the tissue or budding out into the lumen. **Below:** Early stages (ES), intermediate stages (IS), and advanced stages (AS) present in the digestive tubules. AS contains refringent granules (grey/green colouration).

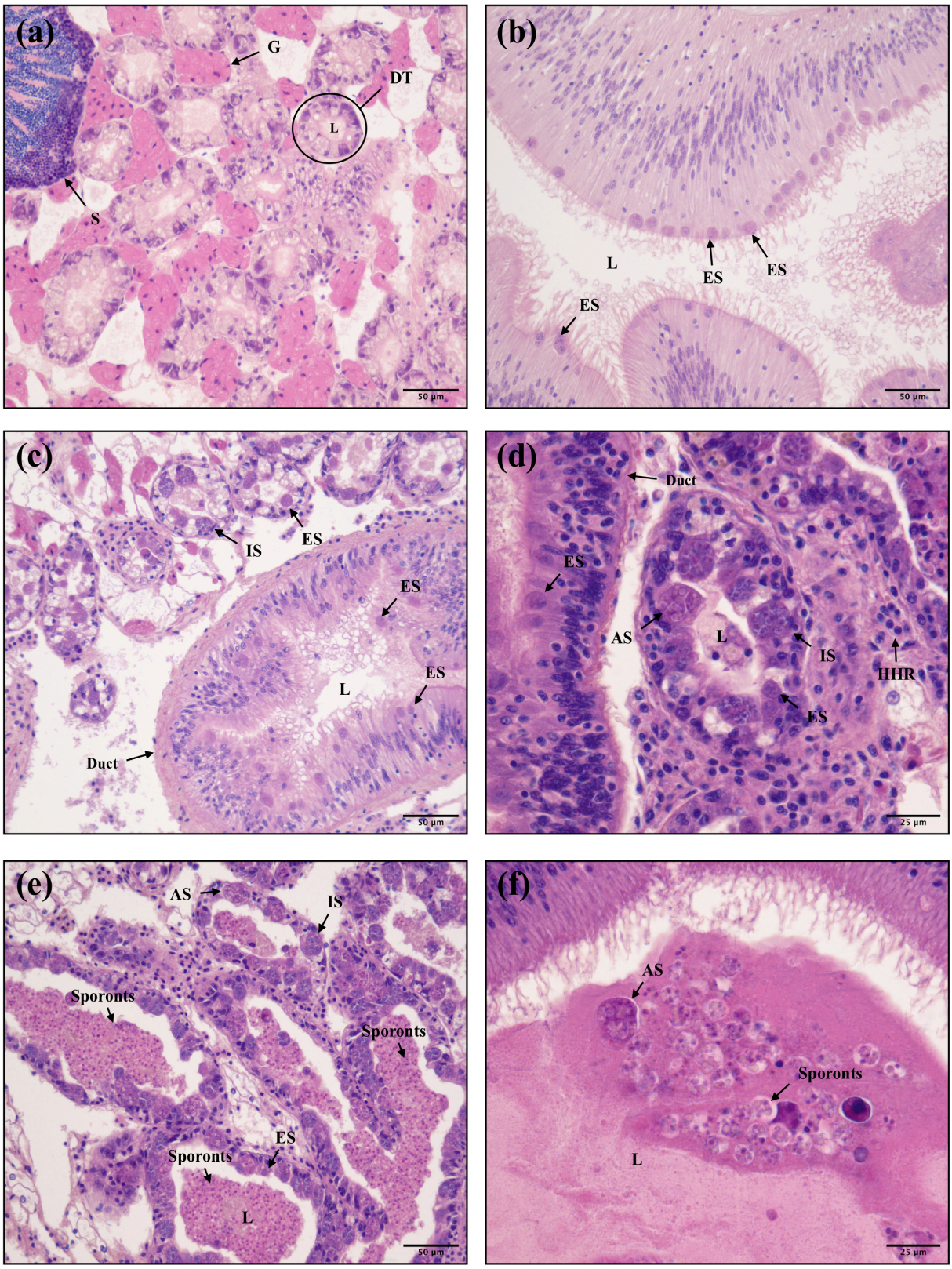

Figure 9: Marteilia pararefringens sp. nov. infecting *Mytilus edulis*. LM, HES staining. (a) Healthy, mature male with sperm (S) and adipogranular tissue (storage tissue; G). One digestive tubule (DT) is encircled, with a prominent lumen (L). (b) Early stages (ES) of the parasite (arrows) in the apical border of the stomach epithelium. L: lumen of the stomach. (c) Early stages of the parasite (arrows) infecting the duct. Early stages of sporulation (ES) can be seen in the ducts and digestive tubules (DT) and intermediate stages of sporulation (IS) only in DT. (d) Advanced stages of sporulation (AS) present in the digestive tubule. IS and ES are also present. Host haemocytic reaction (HHR) surrounds the digestive tubule. ES present in duct. (e) Sporonts present in the lumen of the digestive tubules. ES , IS, and AS present in digestive tubules. (f) Lumen of stomach, L, with sporonts and an AS containing sporonts. Higher magnification so that it is possible to see the sporonts.

Figure 10 and Figure 11 shows the pattern between the PIDT and MNPIDT, PES, PIS, and PAS that characterize the progression of the infection. When pooling the mussels based on PIDT into 20% intervals (e.g. 0%-20%, 20%-40%, etc.), then MNPIDT significantly increased as the infection progressed to more digestive tubules ($R_s = 0.85$, $p < 0.001$). The development of the parasite was quantified using ES, IS, and AS (and their relative percentages PES, PIS, and PAS). The PES stayed relatively high throughout the progression of the parasite, but consistently decreased as the infection progressed. PIS and PAS consistently increased as the infection progressed, both peaking at 80-100% PIDT. PES significantly decreased as the infection progressed ($R_s = -0.79$, $p < 0.001$). The PIS and PAS tended to increase as the infection progressed. IS was first present at 0-20% PIDT. PIS peaked at PIDT 80%-100%. The change in PIS was highly significant ($R_s = 0.79$, $p < 0.001$). Advanced stages first appeared at 40%-60% PIDT. PAS peaked at PIDT 80%-100%. The change in PAS was highly significant ($R_s = 0.70$, $p < 0.001$).

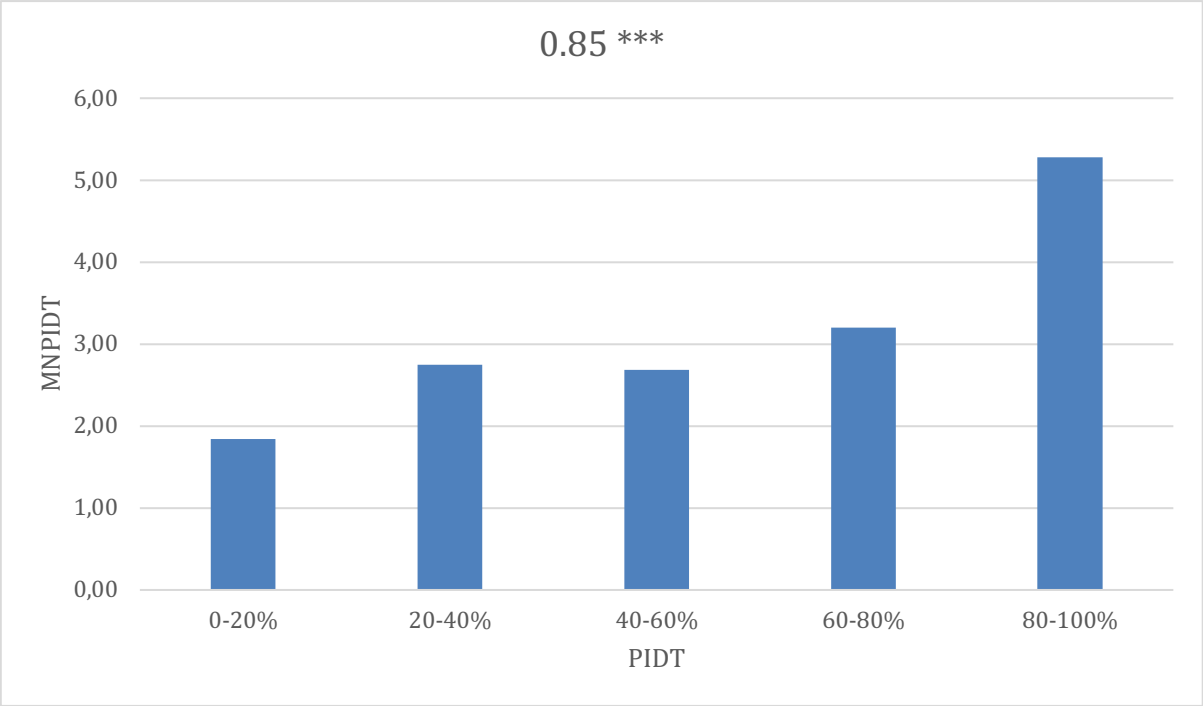

Figure 10: The relationship between the mean number parasites per infected digestive tubule (MNPIDT) and the percentage of infected digestive tubules (PIDT) in *Mytilus edulis* infected by *Marteilia pararefringens*. Infection progression is quantified by the PIDT on the x-axis. Mussels were pooled based on PIDT independent of sampling period, i.e. mussels in the 0% < PIDT ≤ 20% interval could be from August, October or November. Number above the graph represents the Spearman rank correlation coefficient. Significance level ***p < 0.001

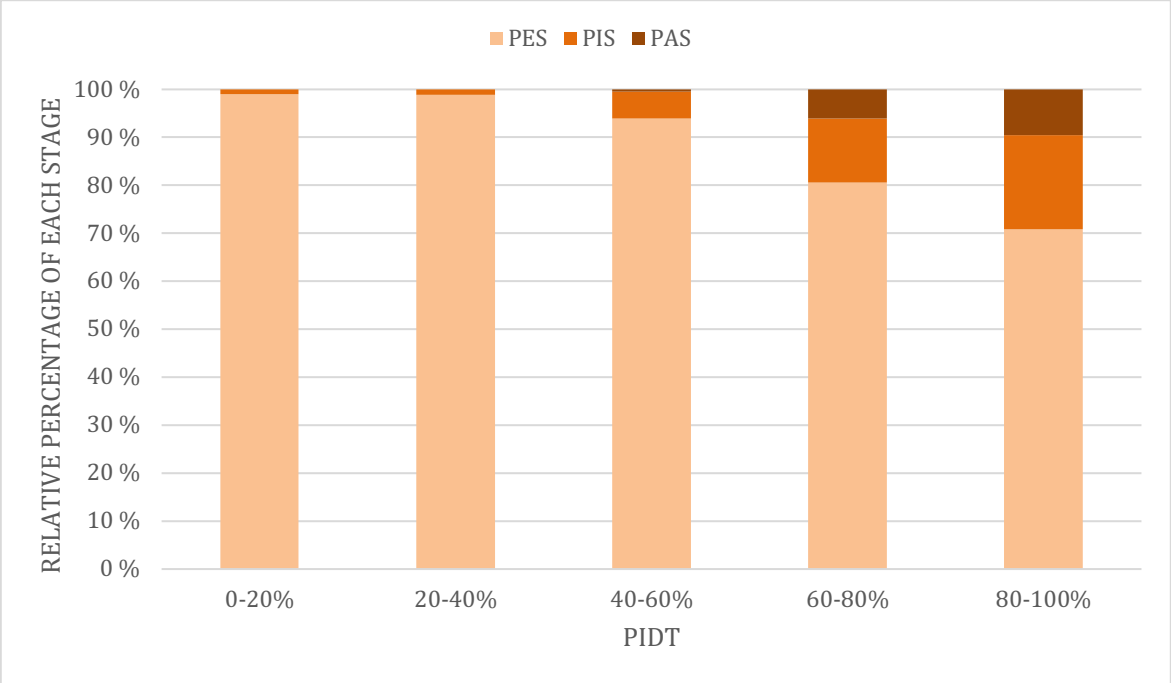

Figure 11: The relationship between percentage of the different stages (PES: early stage; PIS: intermediate stage; PAS: advanced stage) of *Marteilia pararefringens* as the percentage of infected digestive tubules (PIDT) in *Mytlis edulis*. Infection progression is PIDT on the x-axis. Mussels were pooled based on PIDT independent of sampling period, i.e. mussels in the 0%-20% interval could be from August, October or November. Spearman rank correlation coefficients for each varable: PES = -0.79, PIS = 0.79, PAS = 0.70. Significance level for each was ***P < 0.001

Histopathological changes varied with intensity, with focal necrosis of the digestive tubules in light cases and multi-focal necrosis and complete degeneration of the digestive tubules apical membrane in heavy infections. The host haemocytic reaction (HHR) consisted of infiltration in the connective tissues between digestive tubules, ducts, and stomach (Figure 12). The correlation between intensity score and HHR was highly significant (ANCOVA, $F_{1,75} = 60.0$, $p < 0.001$). Mean HHR increased as the infection intensity increased and plateaued after reaching intensity score 4 (Figure 13). Several mussels were infected with other parasites such as trematode larvae (Figure 12e) or crustaceans, with heavy haemocyte infiltration in some individuals.

The prevalence of *M. pararefringens* in the stomach epithelium and ducts was not significant between intensity scores (FET, $n = 59$, $p > 0.05$) (Figure 14).

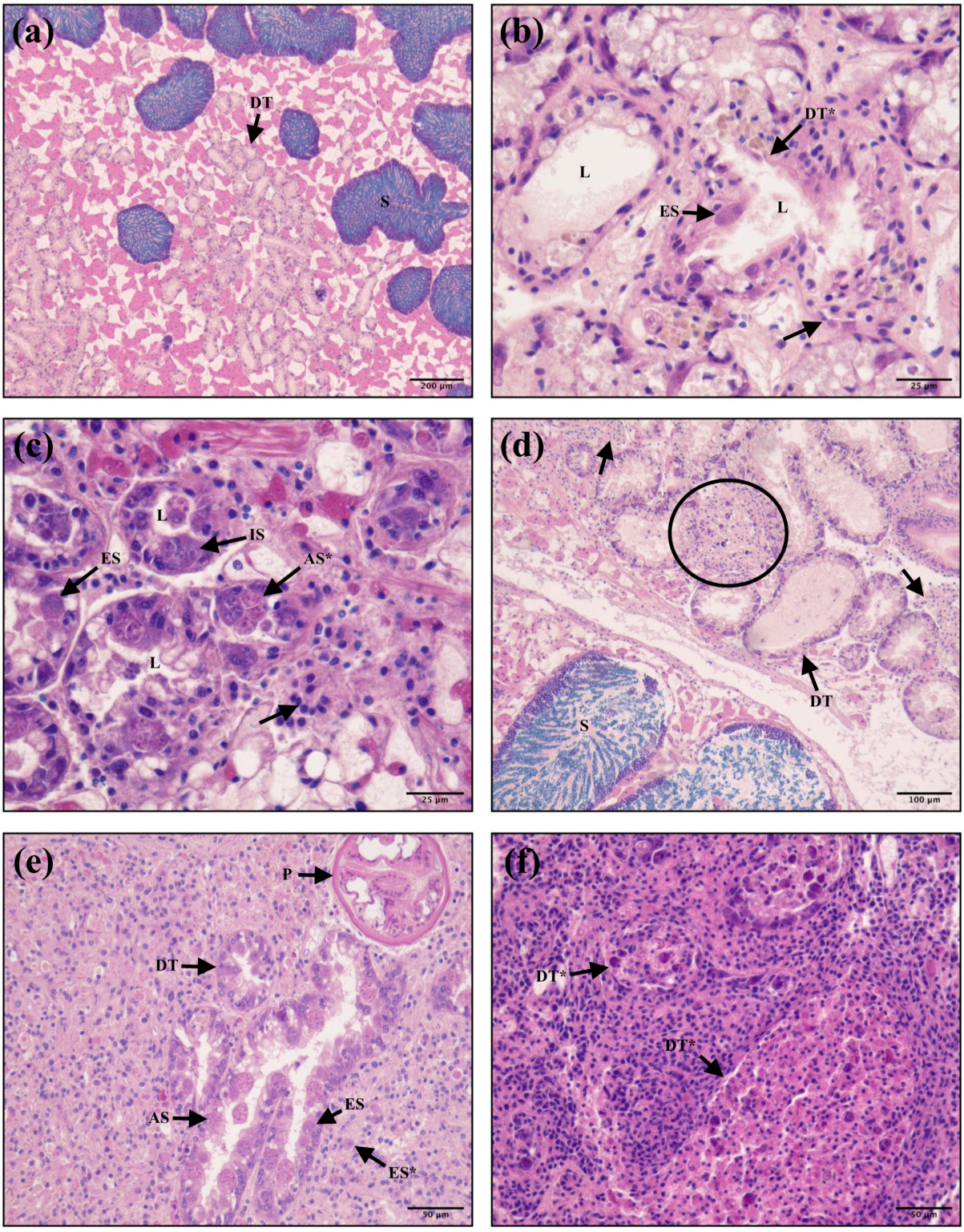

Figure 12: The host haemocytic reaction (HHR) of *Mytilus edulis* infected with *Marteilia pararefringens*. **(a)** Healthy, mature male from May 2018. The tissue has adipogranular tissue (storage tissue; eosin-stained) and sperm cells (S) interspersed with digestive tubules (DT). **(b)** A light infection of *M. pararefringens* at the early stage of sporulation (ES). The infected digestive tubule is degenerate with some haemocyte infiltration present (arrow), with an intensity of 1. L: lumen of digestive tubule. **(c)** A moderate infection of *M. pararefringens*, with ES, intermediate stage (IS), and advanced stage of sporulation (AS*: IS and AS present in same primary cell). Some haemocytic reaction is present (arrow), with an intensity score of 1. L: lumen of digestive tubule. **(d)** A necrotic digestive tubule with haemocyte infiltration (encircled), with healthy, non-infected digestive tubules (DT) surrounding it. Haemocyte infiltration is present in the surrounding tissue as well (arrows). HHR intensity: 2. **(e)** Heavy haemocyte infiltration surrounding the digestive tubules (DT). No healthy tissue left. The DT are infected with primarily advanced stages of *M. pararefringens* (AS), with some intermediate stages (not marked) and early stages (ES). Some ES are also seen scattered in the surrounding tissue (ES*), inevitably from neighbouring necrotic DTs. P: trematode parasite commonly found in *M. edulis*. **(f)** Completely degenerated tissue. No normal digestive tubules left (DT*). Heavy haemocyte reaction. HHR intensity: 3.

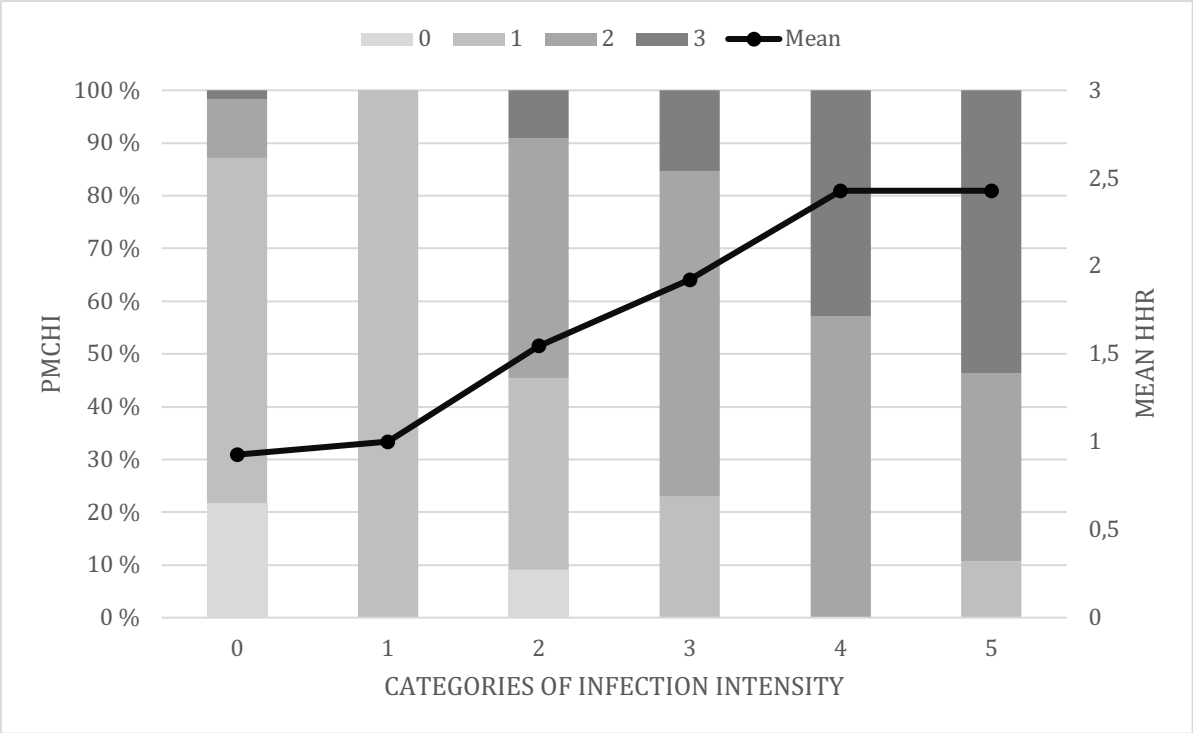

Figure 13: Mytilus edulis host haemocytic reaction with varying intensity scores from May 2018 to November 2018. Individuals were pooled based on infection intensity, not sample period, i.e. each category contains mussels from May to November. PMCHI: Percentage of mussels in each category of haemocytic infiltration.

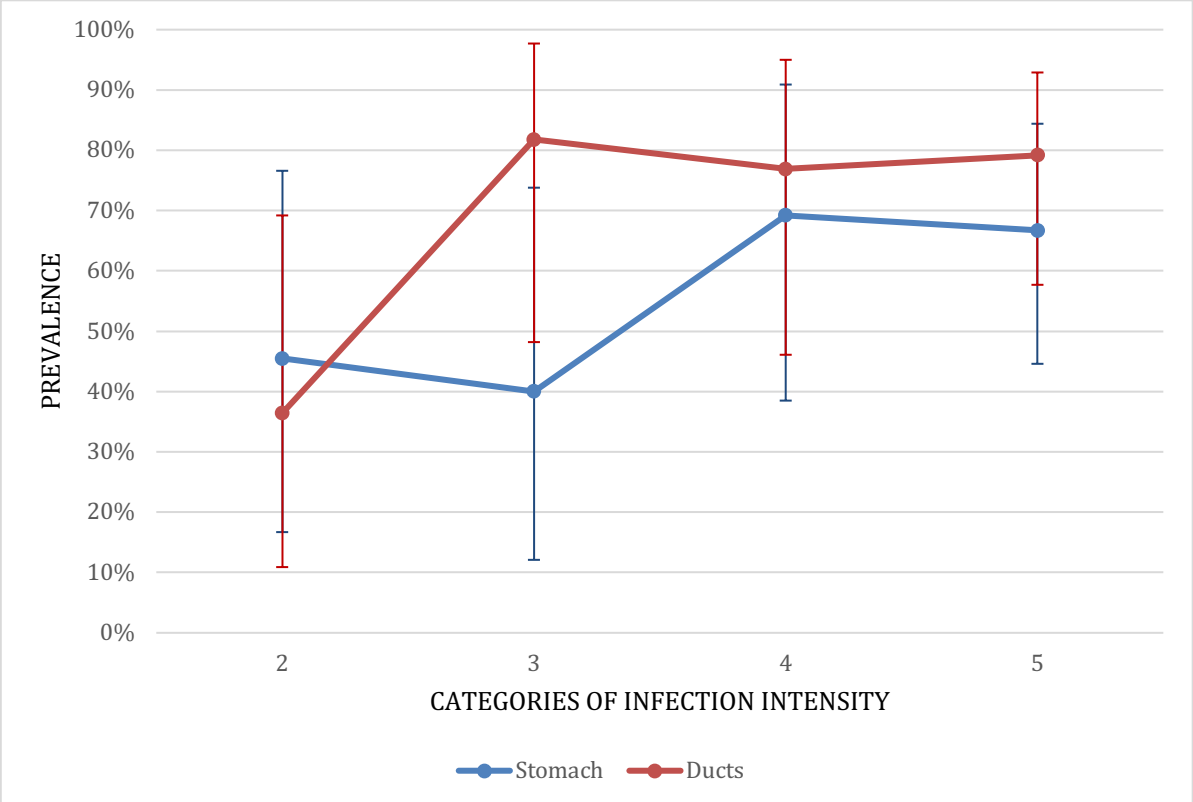

Figure 14: The presence of *Marteilia pararefringens* in the stomach and ducts in relation to the intensity of infection. Intensity scores 0 and 1 (infection only in stomach) not included. 95% binomial confidence interval.

3.1.2 Temporal patterns of the infection

Figure 15 shows the prevalence in each sampling period based on PCR and histological screening. Figure 16 shows a selection of mussels from each sampling period. The earliest evidence of *M. pararefringens* was detected in July through PCR analysis, but no evidence of infection was detected during histological screening at this time. The earliest confirmation of infection by histology was detected in late August, three months after cohabitation began. PCR prevalence increased significantly during the sampling period from May to July (FET, $n = 60$, $p < 0.001$) and July to August (FET, $n = 59$, $p < 0.01$). Sample prevalence did not increase significantly after August. Peak prevalence based on histological detection of the parasite occurred in August at 90%, a highly significant difference from July when no parasites were detected (FET, $n = 59$, $p < 0.001$). Prevalence remained high thereafter. No mussels were infected in the second deployment started in October that lasted until March 2019.

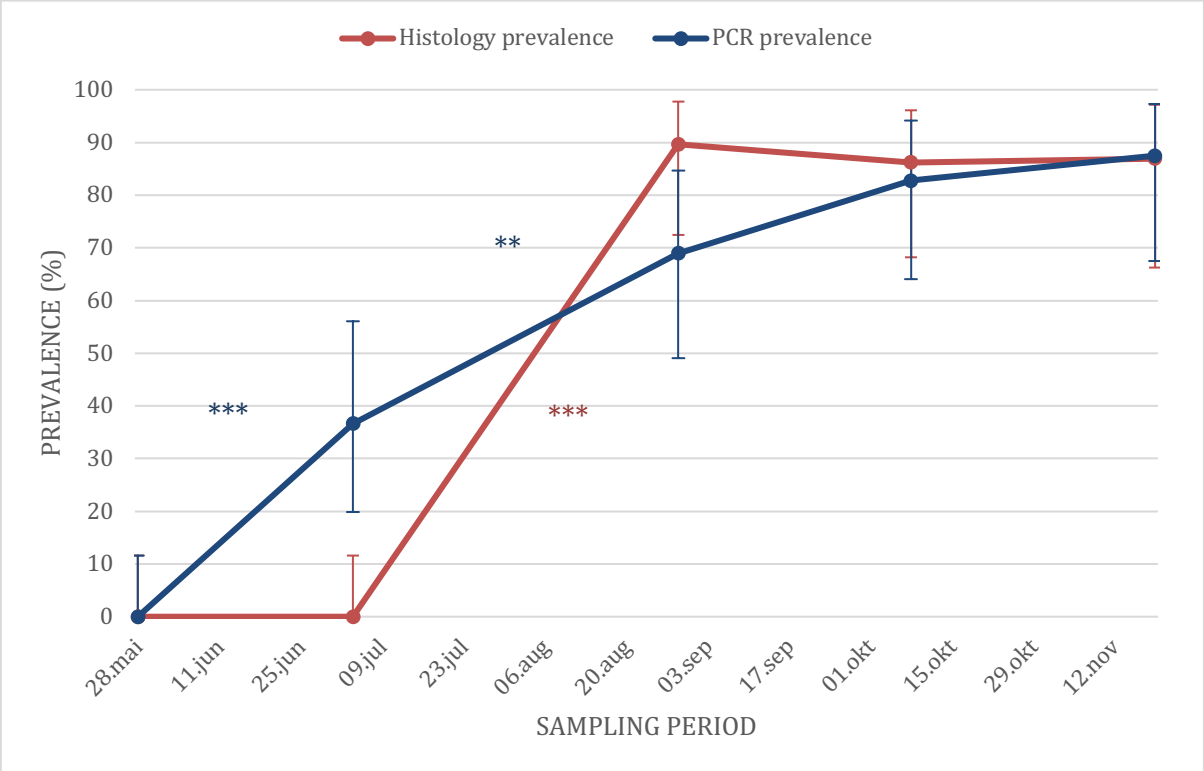

Figure 15: Mytilus edulis infected by *Marteilia pararefringens*. Prevalence based on PCR and histological data is shown. First cohort of 250 mussels deployed in May 2018 until final sampling in November 2018. Mussels form the second cohort from October 2018 to March 2019 not included as these were never infected by the parasite. The healthy mussels were gathered from Rogøysund and placed in oyster baskets and sampled every six weeks. Error bars represent 95% binomial confidence intervals. Significant steps indicated, **p < 0.01; ***p < 0.001.

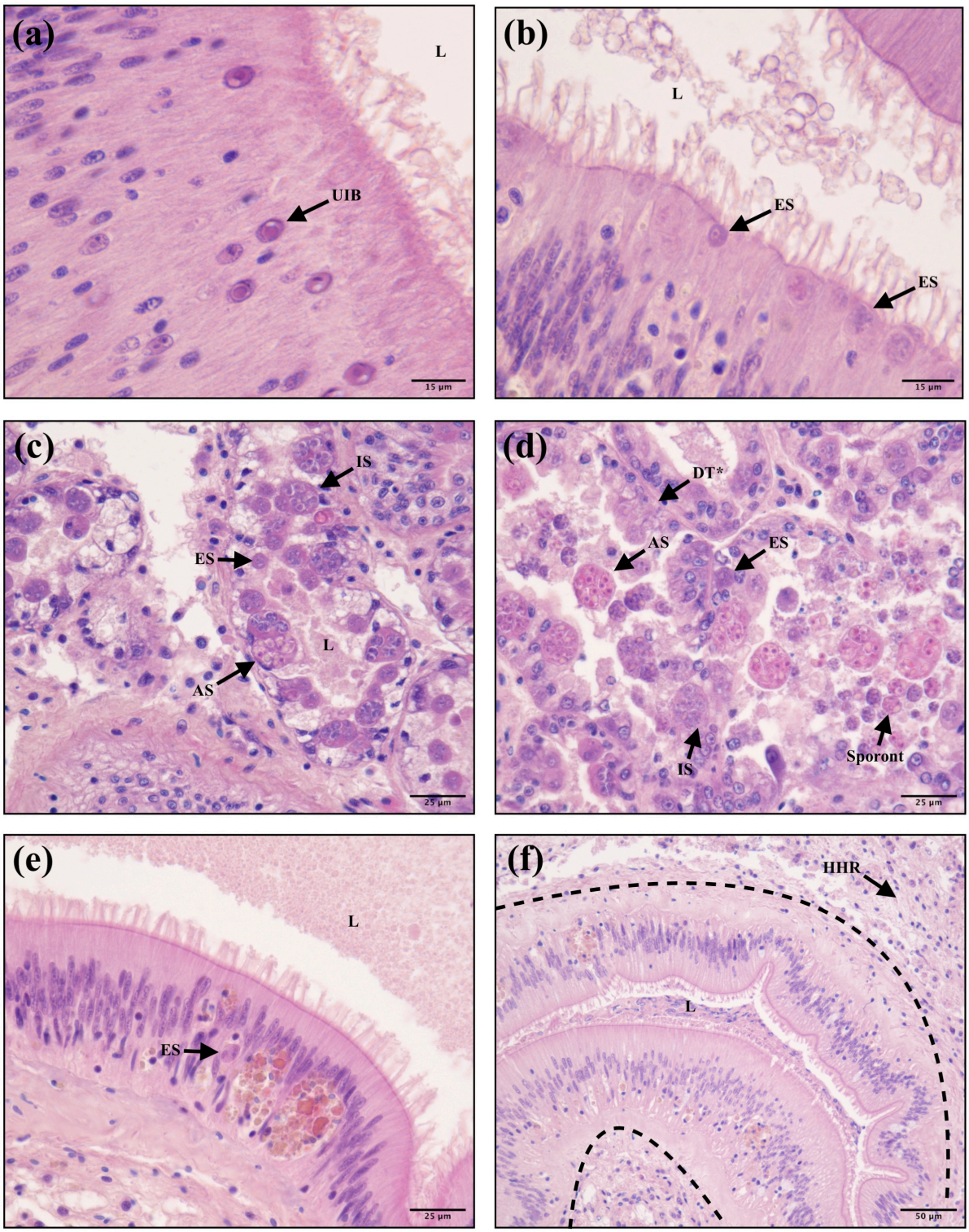

Figure 16: Marteilia pararefringens infection in *Mytilus edulis* from May 2018 to November 2018. LM, HES staining. (a) July sample. Unknown Intracellular Bodies (UIB) found in the stomach epithelium of several individuals. First assumed to be primary stages of *M. pararefringens* as PCR results showed 11 mussels positive for the parasite, but did not match literature description of the primary stages of other known *Marteilia* spp. L: lumen of stomach. (b) Stomach epithelium. Early stages (ES) of *M. pararefringens* were found in several mussels in August, October, and November. L: lumen of stomach. (c) Early stages (ES), intermediate stages (IS), and advanced stages of sporulation (AS) in August sample. Refringent bodies can be seen in the AS as grey-white circles. L: lumen of digestive tubule. (d) October sample. Identical view could be seen in November sample as well. ES, IS, and AS present in partially destroyed digestive tubule (DT*). Sporonts could be seen in the lumen of the digestive tubules. Free AS were also present in the lumen. (e) November sample. ES found in stomach epithelium of several mussels. L: lumen of stomach epithelium. (f) The intestine (dotted line) was never infected with *M. pararefringens*, independent of intensity of infection. L: lumen of intestine; HHR: host haemocytic reaction.

August 2018 saw a rapid infection rate of the mussels. All stages (ES, IS, AS; Figure 18) were present in the sample but varied in frequency within each individual. The PES, PIS, and PAS was 74.6%, 20.0%, and 5.4%, respectively. In several individuals, some spores could be seen in the lumen of the digestive tubules (Figure 9). The correlation between PIDT and PES, PIS, and PAS was significant for the August sample ($n = 24$; PES: $R_s = -0.85$, $p < 0.001$; PIS: $R_s = 0.85$, $p < 0.001$).

The PIDT peaked in the October sample, at 65%. The PAS also peaked at 11%, while both PES and PIS was 70% and 20%, respectively. The correlation between PIDT and PES, PIS and PAS was still significant ($n = 21$; PES: $R_s = -0.61$, $p < 0.004$; PIS: $R_s = 0.66$, $p < 0.002$; PAS: $R_s = 0.56$, $p < 0.01$). The PIDT decreased to 48% in the November sample. The proportion of the different stages remained similar to in October (PES 77%, PIS 13%, PAS 11%), and the pattern with a negative correlation between PIDT and PES, and positive correlations of PIDT with PIS and PAS persisted ($n = 17$; PES: $R_s = -0.73$, $p < 0.001$; PIS: $R_s = 0.73$, $p < 0.001$; PAS: $R_s = 0.57$, $p < 0.002$).

As PIDT increased, so did the mean number of parasites per section of infected digestive tubules (MNPIDT). The correlation between PIDT and MNPIDT between the sample periods was significant (Aug: $n = 24$, $R_s = 0.94$, $p < 0.001$; Oct: $n = 21$, $R_s = 0.75$, $p < 0.001$; Nov: $n = 17$, $R_s = 0.70$, $p = 0.002$). Number of mussels with infections in the stomach epithelium significantly increased (FET, $n = 42$, $p < 0.001$) and in the ducts significantly increased (FET, $n = 43$, $p < 0.01$) between October and November (Figure 19). Intensity score varied between mussels in each sample group (Figure 17). Infection intensity did not significantly change between sample periods (ANOVA test, intensity score 0 omitted, $F_{2,66} = 2.68$, $p = 0.076$).

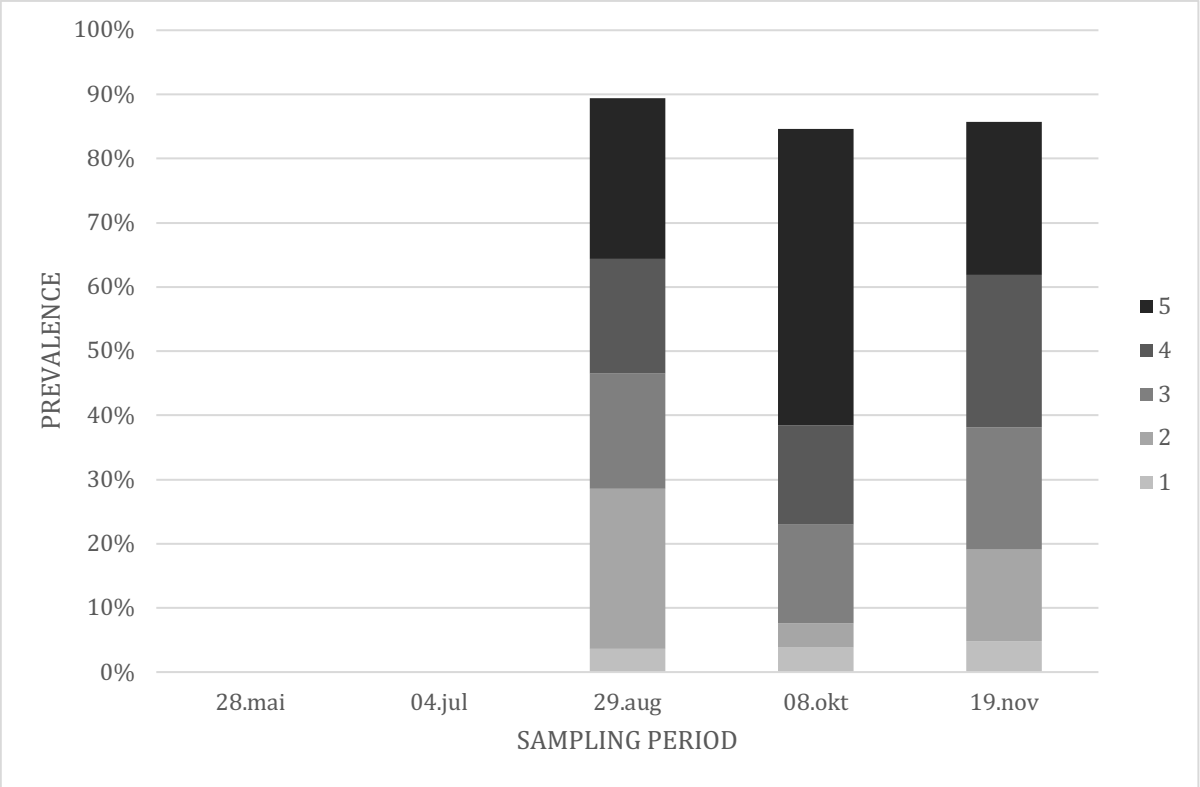

Figure 17: Marteilia pararefringens prevalence and infection intensity during the culture period from May to November for deployment 1. The scale of intensity 1-5 is based on the location and infection degree. 1: light infection - parasite confined to the stomach epithelium; 2: moderate infection – the percentage of infected digestive tubules (PIDT) < 10%; 3: heavy infection – 10% < PIDT < 50%; 4: heavy infection – 50% < PIDT < 90%; 5: heavy infection – PIDT > 90%.

$PIDT = \frac{IDT}{IDT+NDT}$; IDT: infected digestive tubule; NDT: non-infected digestive tubule. $N^{Total}_{Mussels} = 141$, $N^{Total}_{NDT} = 5715$, $N^{Total}_{IDT} = 2274$.

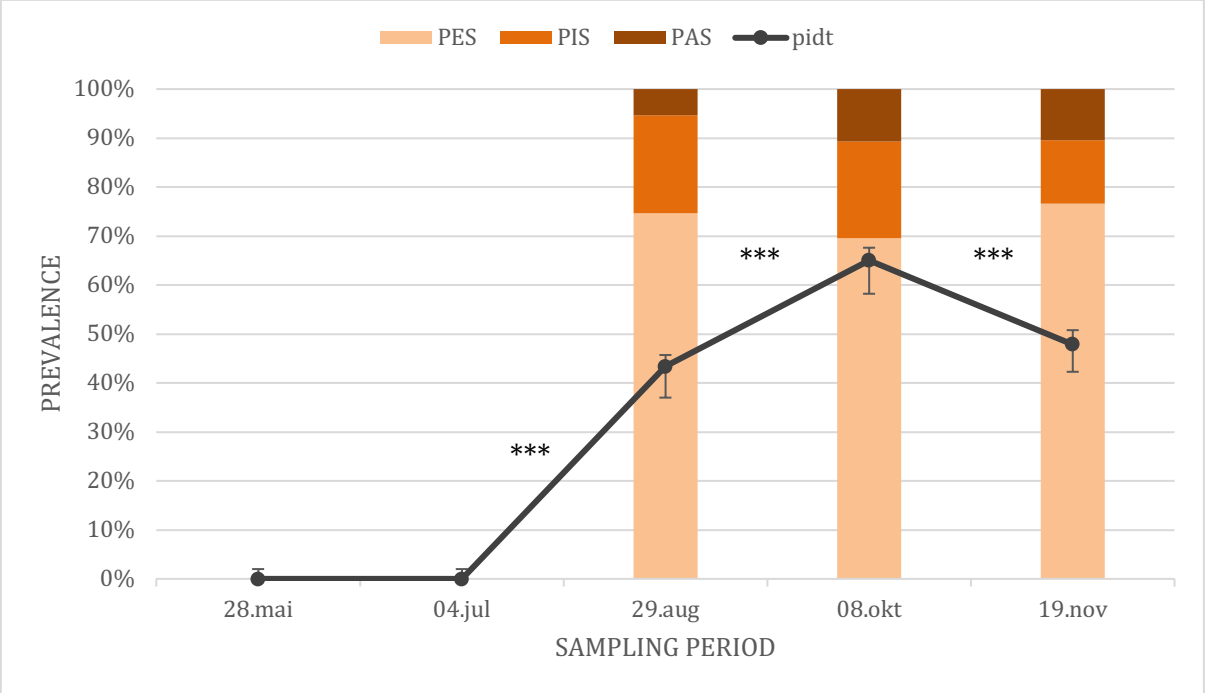

Figure 18: Marteilia pararefringens percentage of infected digestive tubules (PIDT) compared to the ratio of percentage of early stage (PES), intermediate stage (PIS), and advanced stage of sporulation (PAS) per month. The PIDT is based on the number of infected digestive tubules (IDT) versus the total number of infected and non-infected digestive tubules (NDT). The PIDT was calculated for each individual by counting the NDT and IDT in 10 randomly selected fields at 400x magnification and taking the percentage. Total number of mussels for each month: May = 30, July = 30, August = 29, October = 29, November = 23. Total number of NDT: May = 1603, July = 1920, August = 1979, October = 1309, November = 1178. Total number of IDT: May and July = 0, August = 859, October = 1309, November = 564. 95% binomial confidence interval used as error bars for PIDT.

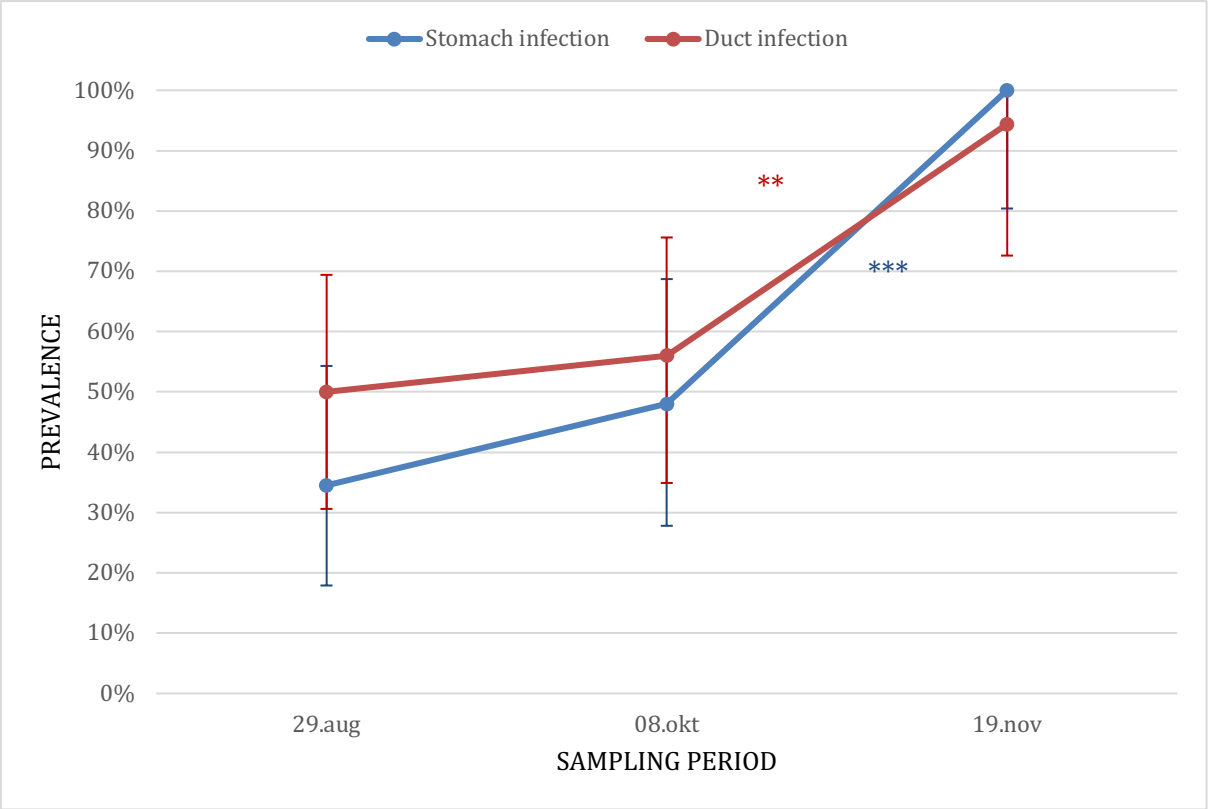

Figure 19: The relationship of prevalence in stomach epithelium and ducts in relation to sampling period. There was an insignificant increase in the prevalence of *Marteilia pararefringens* in the stomach epithelium and ducts from August to October. The was a highly significant increase in both infection sites from October to November. Sampling period in May and July are omitted as these were not infected based on histological screening. 95% binomial confidence intervals. Significance level **p < 0.01, ***p < 0.001

The host haemocytic reaction varied between and within the samples, with the mean highest values for the duration of the study corresponding to the period of infection (Figure 20). Preliminary testing showed that the host haemocytic reaction correlated with intensity in all months ($0.60 < R_s < 0.66$, $p < 0.001$-0.002). Therefore, intensity was included as a covariate when examining temporal changes in host haemocytic reaction using ANCOVA. As expected, intensity showed a highly significant effect on host haemocytic reaction (ANCOVA, $F_{1,75} = 60.0$, $p < 0.001$). Accounting for intensity, the variation in host haemocytic reaction August to November was not significant (ANCOVA, $F_{2,75} = 2.91$, $p = 0.061$).

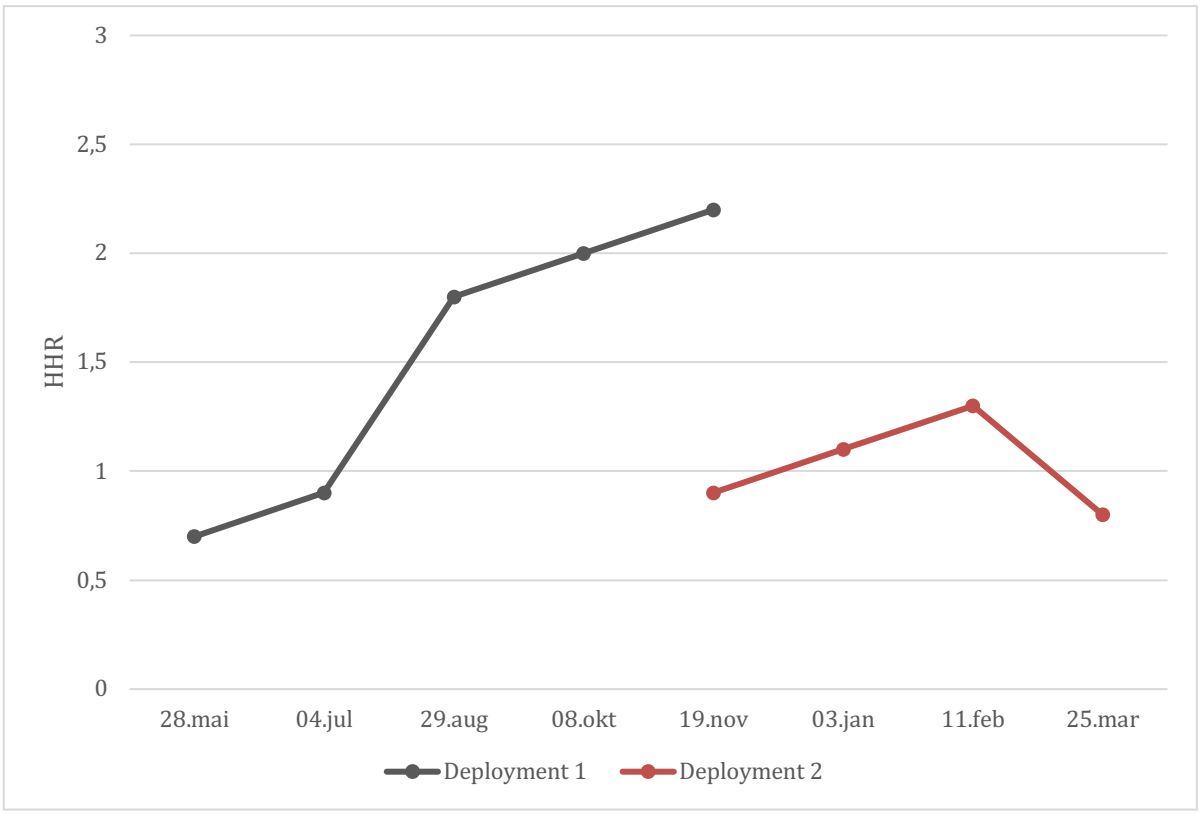

Figure 20: Host haemocytic reaction of two deployments of 250 mussels, *Mytilus edulis*, each. First group was deployed in May 2018. 30 mussels were sampled every six weeks. Second group was deployed in October (only PCR sample –no host haemocytic reaction recorded) to see whether these could be infected during the sporulation phase of *M. pararefringens* in the first group. There was no transmission of the parasite in the second deployment.

3.2 Cohabitation trial in the laboratory

A total of 60 mussels were sampled from the laboratory trials to see whether transmission of the parasite could occur without an intermediate host. One polychaeta was found during sampling of infected mussels, negative for *M. pararefringens.* One mussel from the healthy group was positive for *M. pararefringens* using PCR analysis but showed no clinical signs of infection.

The infected donor mussels from Aga had a prevalence of 78%, with a mean PIDT of 28% and an average intensity of 2.3. This indicates a moderate to heavy infection. The PES, PIS and PAS was 53%, 30% and 17%, respectively. Spores were found in the lumen of some individuals. The average host haemocytic reaction was 1.6, indicating a light to moderate reaction. PCR analysis showed a prevalence of 87%.

The healthy mussels from Rogøysund were negative for *M. pararefringens*, with the exception of one individual. No infection was detected based on histology. There were some host haemocytic reactions, with an average of 1.3.

3.3 Fauna samples

Fauna samples were PCR analysed at CEFAS, Weymouth. A total of 59 shrimp, 22 polychaetes, and 14 vials of plankton were analysed.

Table 5: Fauna samples tested for the presence of *Marteilia pararefringens* using PCR analysis. Positive results were verified to be *M. pararefringens* by testing for the ITS-region

Group/Species	**2018**							
	May		July		August		October	
	Sample size	Prevalence	Sample size	Prevalence	Sample size	Prevalence	Sample size	Prevalence
Shrimp/ *Palaemon* sp.*	16 ind.	0	16 ind.	1+	7 ind.	0	20 ind.	0
Plankton	3 vials	0	4 vials	4	5 vials	5	2 vials	1
Polychaeta					9 ind.	0	13 ind.	0

**P. elegans* or *P. adspersus*

One shrimp of the 59 tested was positive for *M. pararefringens* in July. The rest were negative. Of the 14 vials of zooplankton sampled, 10 were positive: all 4 were positive in

July, all 5 were positive in August, and 1 of 2 was positive in October. Analysis of taxa was also performed, but not divided into sampling period (Appendix C). No polychaeta were positive for *M. pararefringens.*

No fauna was sampled from January to March.

3.4 Restriction Fragment Length Polymorphism

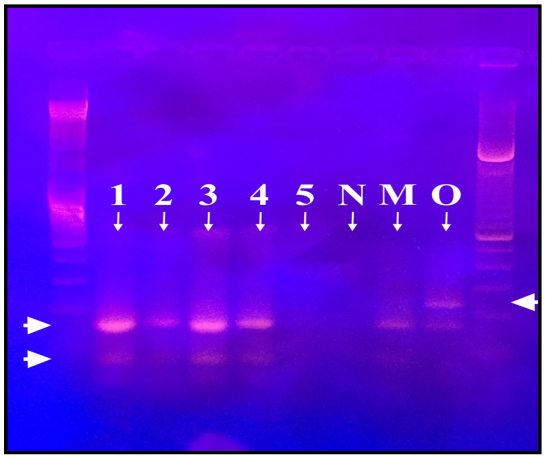

Figure 21: Restriction fragment length polymorphism (FRLP, *HhaI* restriction site) analysis of PCR products (ITS region) demonstrating the indentifcation of *Marteilia* spp. Position N is the negative control. M is the positive control for *Marteilia pararefringens,* characterized with one band at 156-bp and one (weak) at 68 bp (left arrows). O is the positive control for *M. refringens*, characterized with a band at 226 bp (right arrow) and one at 156 bp. Position 1-3 indicate positive individuals from each sampling group (August, October, and November), with a RFLP profile identical to *M. pararefringens*. Position 4-5 indicate positive individuals from each group in the transmission trial in the laboratory at the Institute for Marine Research, Bergen. 4 came from the infected group from Aga and the RFLP profile is identical to *M. pararefringens*. 5 is the weakly positive mussel from the healthy group from Rogøysund in the cohabitation trial. The sample was too weak to be seen in RFLP analysis.

PCR-RFLP analysis was performed on one positive mussel per positive sampling group (Figure 21). Mussels from August, October, November, and the positive group in transmission trial in the laboratory were infected with *M. pararefringens.* RFLP was unsuccessful at amplifying the positive mussel from the healthy group in the transmission trial in the laboratory.

4 Discussion

The detection of *Marteilia pararefringens* infecting *Mytilus edulis* in an oyster pond at Aga, Bømlo, Norway has provided a unique opportunity to study this parasite in a semi-closed system, similar to the claires in France. Through a cohabitation trial with healthy mussels at Aga, the transmission window of *M. pararefringens* was found to be between July and August. Based on the presence of advanced stages in the digestive tubules and sporonts in the lumen of the digestive tract, sporulation of the parasite was determined to occur from August to November. Transmission of the parasite did not occur from October to March.

Transmission of the parasite from infected donor mussels to healthy mussels in the laboratory was unsuccessful, indicating the need for an intermediate host. Plankton samples were positive from July to October. One shrimp was positive but was most likely due to accidental ingestion of the parasite from feeding on dead, infected mussels. No histology has so far been performed to verify whether the presence in the fauna samples were actual infections.

4.1 *M. pararefringens* infection in *M. edulis*

The development and sporulation of *M. pararefringens* is consistent with the description of the phylum Paramyxae (Desportes & Perkins 1990). The histological description of this parasite coincide with those described for *M. maurini* (Comps et al. 1982) including type host (Auffret & Poder 1983). However, specific ultrastructural characteristics could not be determined as it requires electron microscopy (Kerr et al. 2018).

The life cycle of *M. pararefringens* has not been conclusively demonstrated, but the development in the host *M. edulis* is described. Transmission from a still unknown source occurs is possible from July, as evidenced by positive PCR results. Infections could not be conclusively verified by histology in this period. This could have been due to either a low infection intensity or a non-infection where *M. pararefringens* spores were present in the lumen of the digestive tract either free-living or within another organism (such as plankton). In July the first positive results from the fauna samples occurred, with one shrimp and all plankton samples being PCR positive. If the intermediate host is a copepod species, as suggested in *M. refringens* (Carrasco et al. 2007, 2008; Berthe et al. 2004), sporulation from this intermediate host could occur from this period onwards. The fact that histological

screening of mussels could not verify the presence of the parasite indicates that this period is at the beginning of the life cycle.

The first evidence for infection of the parasite in mussel tissue was in August, with a sample prevalence 90% for 28 mussels. Site of infection varied, with some mussels only showing an infection in the stomach epithelium, while others were heavily infected in stomach epithelium, ducts, and digestive tubules. Villalba et al. (1993) described the first signs of infection of *M. refringens* in *M. galloprovincialis* to occur in the host's stomach epithelium, where the parasite persists for all infected individuals. However, in this study *M. pararefringens* infection in the stomach epithelium in *M. edulis* in the present study was only present in 39 of the 64 infected individuals with the stomach present. This might be explained one of two ways: (1) either low intensities manifests as infections in the stomach epithelium, or (2) heavily infected mussels become overwhelmed, leaving the stomach epithelium more prone to new infections. However, the number of mussels infected with the parasite in the stomach did not vary between infection intensity of the digestive tubules, which means that infection intensity does not correlate with stomach infection. That being said, correlation between prevalence of the parasite in stomach epithelium (and ducts) and sampling period was significant.

Surprisingly, the most severe infections in the stomach epithelium were most often found in the November samples. If one assumes that the development of *M. pararefringens* is somewhat similar to that of *M. refringens,* where the progression of the parasite starts from the stomach and develops in the digestive tubules, the high prevalence of the parasite in the stomach epithelium could be the result of new infections occurring just before the intermediate host disappears for the winter. The infection of the parasites in the stomach could lay dormant as the temperature decreases, beginning development only when the temperature increases again in the spring of the following year. Since all mussels from the first deployment were depleted in November and the mussels in the second deployment in October were never infected, no data could be gathered to elucidate the pathology during the winter.

It seems as though maturation of *M. pararefringens* does not occur in the stomach epithelium – only early stages were found in this organ, regardless of sampling period. Such results have also been described in *O. edulis* and *M. galloprovincialis* infected with *M. refringens* (Grizel,

1979; Villalba et al. 1993), *S. glomerata* infected with *M. sydneyi* (Kleeman, Adlard & Lester 2002), and *C. gigas* infected by *M. refringens* (Montes et al. 1998). It is difficult to ascertain whether the stomach epithelium is an 'accidental' infection site where the primary cells are unable to propagate, if the parasite is able to migrate to the digestive tubules, or if this site allows for extrasporogenic development to increase the infection intensity. Extrasporogenic development is the proliferation of infective stages in other sites not involved in sporulation. In this regard, the stomach epithelium seems to be in a 'grey zone' of whether it can be included in the definition of "sites not involved in sporulation," as some development does occur, but sporulation has never been reported in *M. pararefringens* (or *M. maurini*). Villalba et al. (1993) very rarely reported sporulation stages in the stomach epithelium in *M. refringens* infections in *M. galloprovincialis*.

Extrasporogenic development was first verified in a study on *M. sydneyi* infecting *S. glomerata,* but it was only found in the gills and palp epithelium (Kleeman, Adlard & Lester 2002). *M. refringens* has been detected in the gills of *M. galloprovincialis* (Robledo & Figueras 1995), as well as the gills and labial palps in *M. edulis* (Garcia et al. 2009), but these findings are uncommon and have not been associated with any evidence of extrasporogenic development.

By definition, extrasporogenic development cannot occur in the digestive tubules as these sites are the primary tissues for spore development and maturation. However, a similar process could explain the relationship seen in the mean number of parasites (MNPIDT) in relation to the percentage of infected digestive tubules (PIDT). MNPIDT was strongly correlated with PIDT, indicating that the number of parasites per infected tubule increases as the infection progresses. This progression implies an exponential growth in the number of parasites as more digestive tubules are infected with more parasites (Figure 22). This development could be explained by (1) many new spores that constantly infect the mussels over the duration of the transmission window, or (2) fewer spores infect first and proliferate through merogony (vegetative multiplication), increasing the intensity of infection (Figure 23).

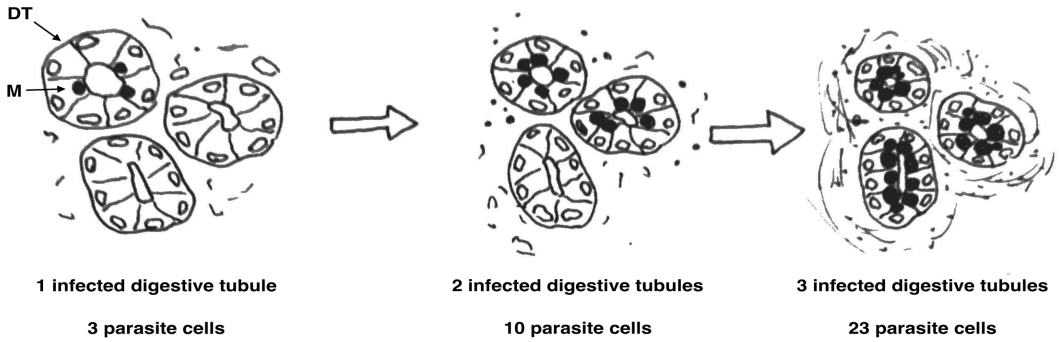

Figure 22: The exponential growth of the number of *Marteilia pararefringens* cells (M) infecting the host *Mytilus edulis* as the infection progresses. First, the infection is limited to a few digestive tubules (DT) with a low abundance of the parasite. In this example, one of three DT are infected with three parasites. As the infection progresses, more DTs are infected by the parasite (usually at different developmental stages). Each infected DT now has on average five parasites. As the progression of the infection reaches its peak, all DTs are infected, with an average number of parasites of ca. 8.

If (1) is true, a constant infection of early stages in the stomach epithelium could be expected as long as there are viable spores in the water column (i.e. in the transmission period). However, this assumes that infective stages always infect the stomach epithelium first, followed by migration to ducts and digestive tubules. The results do not verify this hypothesis as infected individuals were not always infected in the stomach epithelium. In heavily infected individuals where all digestive tubules were infected, several hundred parasites were consistently observed. If hypothesis (1) was valid, it would mean that each parasite would have to come from a different infective spore, and each undergo sporulation. It seems much more likely that the high number of parasites originate from fewer infective spores that replicate after infection of the host. This is consistent with findings

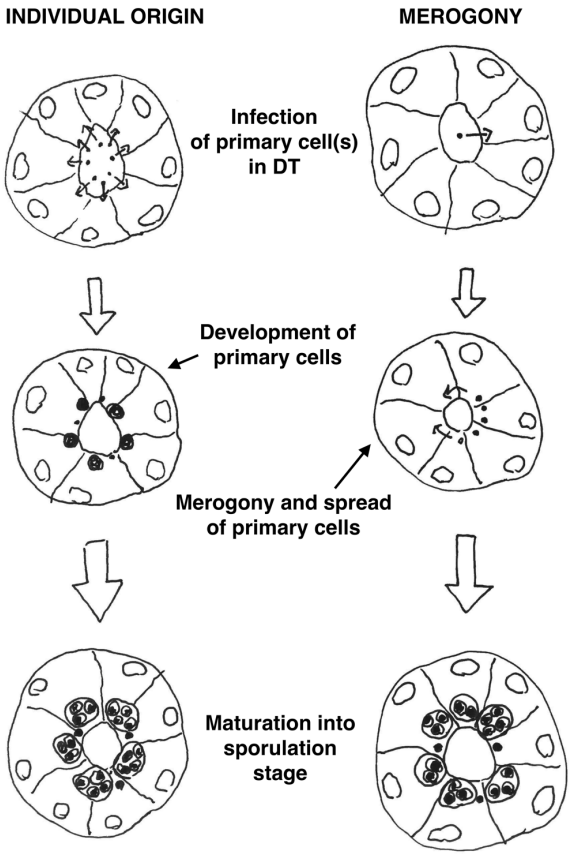

Figure 23: **Left**: Maturation of *M. pararefringens* in a digestive tubule (DT) if each primary cell developed from one unique spore. With high infection intensities, the potentially hundreds of parasites must have developed from as many spores. **Right**: Maturation of *M. pararefringens* if one spore can multiplicate into several primary stages before development occurs. High infection intensities can be achieved by a few spores undergoing vegetative multiplication (merogony)

reported by Villalba et al. (1993). However, no multiplication other than sporogony have been conclusively demonstrated for *Marteilia* spp. by microscopical observation in the target tissues (Carrasco, Green & Itoh 2015).

Sporulation of *M. pararefringens* from its bivalve host occurred from August to November, based on the presence of advanced stages (AS) in the digestive tubule epithelium and spores in the lumina of the digestive tract. One sporulation cycle in a year is in contrast to studies on *M. refringens* by Carrasco et al. (2007), Robledo & Figueras (1995), and Villalba et al. (1993), who all found sporulation occurring twice a year in that parasite. However, temperature seems to have an important role in the development of *Marteilia* spp., which means the relatively colder temperatures present at higher latitudes might only facilitate one sporulation cycle, as seems to be the case with *M. pararefringens.* The occurrence of peak PAS when the infection had reached maximum spread is an effective survival tactic as it increases the chances of the spores reaching a susceptible intermediate host. This reproductive strategy has been described in *M. refringens* in both *M. galloprovincialis* (reported; could be *M. pararefringens*, but no molecular diagnostics were used to verify species) and *O. edulis* (Villalba et al. 1993; Alderman 1979). It seems necessary that some form on initiator is responsible for such high numbers of parasites seen in heavily infected individuals. This might be due to a weak condition of the host facilitating the progression of the infection, but it could also be that the progression of the parasite contributes to the weakening of the host.

Data from the present study showed a correlation between the intensity of infection by *M. pararefringens* and the host haemocytic reaction. Evidence suggests that the host attempts to stop or slow down the progression of the parasite with a light haemocytic reaction. If it fails to stop the spread, the host haemocyte reaction increases as the infection intensity increases, but there seems to be variations between mussels. Several mussels were heavily infected (intensity score 5), but had only a light haemocytic infiltration, while some had a moderate infection (intensity score 2) but had a heavy haemocytic infiltration. This could be explained either by a rapid infection and progression of the parasite that evades the host's immune response, or the host's defences are overwhelmed. Massive haemocyte infiltration has also been reported in *M. galloprovincialis* infected with *M. refringens* (Villalba et al. 1993) and *Marteilia* sp. (Figueras et al. 1991), and variable amounts of haemocytic reaction was observed by Alderman (1979) in *O. edulis* infected by *M. refringens*. The host haemocytic

reaction was concentrated around the digestive tract, including stomach, ducts and digestive tubules. However, It is important to note that the haemocytes also play crucial roles in other processes, such as uptake of nutrients and expulsion of waste, that cause presence around the digestive tract to vary greatly between individuals, especially around the stomach and ducts (Hovaard, Mortensen & Strand 2001, p. 38). It is also important to note that some mussels had heavy haemocyte infiltration due to other causes, such as additional parasite-species and congested non-parasitic organisms (e.g. small crustaceans). These were often found in conjunction with *M. pararefringens* infections. The weakening of the host's defence system due to these accompanying causes could facilitate the spread of *M. pararefringens* in the tissues. However, heavy haemocyte infiltration did not always coincide with *M. pararefringens* infections.

No analysis on whether the presence of foreign bodies had a significant effect on the spread of *M. pararefringens* in mussels was done, but it poses an interesting question: why were some mussels infected (and several to a heavy degree) while others were healthy? Every mussel used in the study came from the same area, Rogøysund, where neither marteiliosis nor any significant mortalities have been reported (Mortensen et al. 2019). There is evidence that suggests naïve bivalves transported from a region free from marteiliosis to one with persistent issues with the parasite results in nearly 100% mortality of the introduced population (Carrasco, Green & Itoh 2015). *M. edulis* from Britain were introduced to Aber Benoit, France in a zone where both *M. refringens* and *M. maurini* (now *M. pararefringens*) are present. The imported mussels were seeing nearly 100% mortality after infection of *M. maurini*, while the local population only experienced low infection levels with no significant increase in mortality rates (Le Roux, unpubl. data; Berthe et al. 2004). A similar case was reported in *O. edulis* transported from Norway to the Thau Lagoon in France (Berthe et al. 2004).

It is apparent that selection of mussels resistant to the parasite occurs in endemic areas, reaching a host-parasite equilibrium that facilitates successful propagation of *Marteilia* without eradicating the host population (Carrasco, Green & Itoh 2015). Obviously, adaptation to local environmental conditions plays an important role in host susceptibility, as shown by Fuentes et al. (2002) who studied the effect genetic origin has on *M. galloprovincialis*, *M. edulis,* and hybrids' resistance to *M. refringens* infection. However, the resilience of mussel populations with long historical association with *Marteilia* sp. does not explain the variation

in infection intensity and prevalence of *M. pararefringens* in the mussels in this study. Hybrids have been shown to be more susceptible to *Marteilia* infection (Fuentes et al. 2002) and *M. galloprovincialis* has been found in Norway (Brooks & Farmen 2013), so principally the mussels used in the present study could be a genetic mix. However, the study by Kerr et al. (2018) analysed mussel tissue from the region and exclusively found *M. edulis*, which could mean the mussels used in the study were most likely not hybrids. That being said, the sample size sent to analysis by Kerr et al. (2018) was small and might therefore not be representative.

Prevalence of the parasite varied within the mussels used in the cohabitant study as well as the natural population present in the area, but no oysters have been infected with the disease since the surveillance program began in 2006. The host distribution mimics that reported from Croatia by Zrnčić et al. (2001) and the west coast of Spain by Figueras & Robledo (1993), where infected mussels cultured side-by-side flat oysters did not transmit the disease to the oysters. This host distribution could be explained by: (1) two species of *Marteilia* that have different host tropisms, (2) a different intermediate host that might not be present is necessary in the transmission of the parasite between the two bivalve hosts, or (3) the flat oysters could be resistant to infection. Kerr et al. (2018) has demonstrated that there are two different *Marteilia* species infecting mussels and oysters, but this does not discredit the relevance of (2) and (3).

4.2 Transmission trial of *M. pararefringens*

The controlled transmission trial in the laboratory at IMR was unsuccessful –none of the 30 healthy mussels sampled of the 100 from Rogøysund became infected, except one that most likely had ingested a non-infective spore.

The mussels from Aga that were presumed infected were taken from Agapollen. 10 mussels were screened from Agapollen in October to verify presence of the parasite and evaluate developmental stage. These were found to be at a similar stage as those in the cohabitation trial in the channel. After the laboratory trial was completed, the donor mussels were verified to be infected with advanced stages of *M. pararefringens* cells in the digestive tubules. The infected mussels seemed to have developed further than those used in the cohabitant study at Aga, with lower fraction PES and higher fraction of PIS and PAS. The reason for this is unknown. Perhaps the temperature in the laboratory was high enough for more advanced stages to develop after transport from Agapollen.

Spores were seen in the lumen of the digestive tubules. This indicates that there should have been sufficient spores in the water column for a potential infection to take place in the healthy mussels. However, this assumption should have been verified by sampling the water column every few days as this could have given an indication of what level of infection pressure the mussels were exposed to.

Even though the infection pressure was not known, the PCR results indicated that there must have been spores circulating in the water column. One individual from the healthy mussel group was PCR positive for *M. pararefringens*. This was not verified by histological screening, but it is not uncommon for prevalence to vary using different methods (Burreson 2008; Aranguren & Figueras 2016). The use of PCR assays do not give an indication of actual infection, but rather a proxy indication of pathogen presence on the basis that they only detect DNA sequences and not actual presence in viable pathogen cells (Burreson 2008). Since the positive PCR result could not be verified by histology, it cannot be concluded that transmission of the parasite was successful.

Studies on the horizontal transmission of *Marteilia* sp. are abundant but have been unsuccessful (Balouet 1979; Grizel 1985; Berthe et al. 1998), which data from this study

supports. The unsuccessful transmission trials have led to the suspicion of an intermediate host (Carrasco, Green & Itoh 2015). The use of advanced molecular techniques have improved the efficacy of finding the distribution of *Marteilia* species in the environment, but the issue with distinguishing infections from non-infections persists (Burreson 2008; Carrasco et al. 2007). However, PCR assays allows for rapid analysis of the prevalence of the parasite in the environment, which can narrow down the search.

4.2.1 The Intermediate Host Conundrum

The intermediate host of any *Marteilia* spp. life cycle has never been conclusively demonstrated, despite numerous papers on the topic. *M. refringens* and *M. sydneyi* have been principal focus points due to their detrimental effect on the oyster industry in Europe and Australia, respectively (Berthe et al. 2004; Adlard & Nolan 2015; Carrasco et al. 2007; Boyer et al. 2013). Horizontal transmission of the parasite has been exhaustively studied by using various methods, including cohabitation, injection and feeding of spore suspensions –all but one unsuccessful (Balouet et al. 1979). Comps and Joly (1980) successfully infected apparently healthy *M. galloprovincialis* from mashed digestive gland tissue from *O. edulis.* No other study has been able to replicate these results (Carrasco, Green & Itoh 2015). The discharge of a horizontal transmission model in favour of a heteroxenous life cycle is widely accepted, but this has not simplified the issue.

It has been suggested that spores require a maturation time in seawater or sediments before they become infective, as Grizel (1985) proposed for *M. refringens.* Research into this hypothesis is lacking, but a study conducted by Wesche et al. (1999) investigated the survivability of *M. sydneyi* spores in different environmental parameters (salinity, temperature, freezing, etc.). It was demonstrated that most spores were dead within 7-9 days, with a maximum longevity of 35 days at ideal environmental conditions (15°C, salinity of 34 ppt) (Wesche, Adlard & Lester 1999). The fact that sporulation occurs in autumn, with the first evidence of infection not appearing before July, necessitates a survival strategy either based on maturation in the environment that solves the detrimental effects of environmental parameters studied by Wesche et al. (1999) or in an intermediate host. Since there is no evidence of any morphological changes attributed to development of the spore outside the bivalve host or any known energy reserve in the sporont that allows survival of the spores until the infection window in the summer, maturation in the environment seems unlikely

(Wesche, Adlard & Lester 1999). However, it should not be omitted that what holds true for *M. sydneyi* might not be transferrable to *M. pararefringens*.

DNA-based screening of fauna samples from oyster beds has become a viable method of finding the distribution of the parasite, but this is a tedious task impeded by the sheer number of species present in intertidal areas (Audemard et al. 2001). Several potential intermediate hosts have been suggested in the *Marteilia* sp. life cycle, including filter-feeding or benthic fish (Roubal et al. 1989); *Crangon crangon* (sand shrimp), *Carcinus maenas* (European green crab), and *Echinogammarus marinus* (marine amphipod; previously *Marinogammarus marinus*) in Dutch waters (van Banning 1979); as well as *Spirorbis* spp. and *Polydora* spp. (polychaete worms), *Pomatoceros triqueter* (tube-building polychaete worm), *Crepidula fornicata* (common slipper shell), *Galathea squamifera* (black squat lobster), and *Liocarcinus puber* (velvet crab) in *M. refringens* endemic areas (Balouet et al. 1979). All these potential hosts failed to reveal the presence of infective stages of *Marteilia* sp. (Berthe et al. 2004).

The problem of species diversity has been somewhat mediated by sampling fauna from oyster ponds (claires) rather than oyster beds. Claires are more suitable for studying the life cycle of *Marteilia* sp. as it greatly limits the number of species that can act as intermediate hosts, from over a thousand to only a hundred (de Montaudouin & Sauriau 2000; Audemard et al. 2001). Several species have been positive for *M. refringens* during PCR screening in the claires in Marennes-Oléron Bay, France, studied by Audemard et al. 2001: *Paracartia grani* (calanoid copepod in the Acartiidae family), *Cereus pedunculatus* (Cnidaria), and *Lineus gisserensis* (Nematoda), among others. Using *in situ* hybridization, the gonadal tissue of *P. grani* was shown to be infected by *M. refringens* (Audemard et al. 2002). Audemard et al (2002) demonstrated that the copepod could be infected by cohabiting oysters infected with *M. refringens*. However, transmission of *M. refringens* from infected copepods to healthy oysters failed (Audemard et al. 2002). The fault might not lie in the hypothesis, but in the design of the experiment. Berthe et al. (2004) has proposed enhancing the inoculum of *M. refringens* by improving the environmental conditions of the copepods so that more can become infected or by increasing the transmission rate from oysters to copepods used in the trials (i.e. increasing the intensity of infection in the copepods to increase the sporulation rate) (Berthe et al. 2004).

The ecology of *P. grani* in oyster ponds and the epidemiology of the disease seem to be consistent with the life cycle of *M. refringens* in Southern Europe (Berthe et al. 2004). The first observations of the copepod in the oyster ponds are in spring and summer, with transmission of the parasite to oysters occurring during the summer, consistent with *P. grani* seasonality (Audemard et al. 2002). Evidence has shown that *M. refringens* can sporulate during spring, most likely developed from the sporangia primordia present through the winter (Boyer et al. 2013; Berthe et al. 2004). It is important to note that no transmission of the parasite occurs during the winter, consistent with the absence of *P. grani* in the water column (Berthe et al. 2004). This is also consistent with results from this study, where the second deployment in October did not become infected by *M. pararefringens*.

The absence of the copepod during the winter is due to dormancy, a trait shared by representative of three copepod taxa. The copepod produces two types of resting benthic eggs: subitaneous eggs, which directly react to unpredictable hostile conditions, e.g. low temperature and desiccation; and diapause eggs, produced before the onset of adverse conditions (i.e. cyclical) and lay dormant until a particular stimulus is achieved, e.g. temperature- or photoperiod-dependent (Boyer & Bonnet 2013). The eggs are located in the sediment (Boyer & Bonnet 2013), which could harbour a reservoir for *Marteilia* during the winter (Berthe et al. 2004).

Since research into the role of *P. grani* has not conclusively demonstrated its role in the life cycle of *M. refringens*, several other zooplanktonic species have been tested and found positive for the presence of *Marteilia* sp., including the copepods *Acartia discaudata, A. clausi, A. italica, Oithona* sp., and *Euterpina acutifrons* (Carrasco et al. 2007). No studies have tested whether these copepods can transmit the parasite to bivalves and diagnostics have been based entirely on nested PCR, which only detects *cul de sac* infections caused by ingestion (Carrasco et al. 2007; Burreson 2008). In France, another *Paracartia* species (*P. latisetosa*) has been found to be infected by *M. refringens*, further demonstrating the potential role of the *Paracartia* genus in the life cycle of this parasite (Arzul et al. 2013). However, all research into the potential role of *P. grani* in the *Marteilia* life cycle has been focused on *M. refringens* in France and Spain, so the question remains: what of *M. pararefringens* sp. nov. and the parasite's northern distribution? Could *P. grani* be a viable intermediate host in Norwegian coastal waters for this parasite species?

P. grani was, in fact, first described by G.O. Sars in oyster ponds on the western coast of Norway (Sars, 1904) and since been found in the coastal regions of the North-eastern Atlantic and North Sea (Boyer, Arzul & Bonnet 2012). Based on the ability to produce resting eggs, and the fact that such dormant stages are generally favourable in high latitudes (Dahms 1995), it is reasonable to suspect that persistent populations of *P. grani* (and other calanoid copepods) are present in oyster ponds in Norway. Their life cycle strategy allows them to lay dormant until favourable environmental conditions arise (Dahms 1995; Annabi-Trabelsi et al. 2018; Boyer & Bonnet 2013). It seems as though *P. grani* is a viable candidate in the life cycle of *M. pararefringens* considering shared geographic distribution and the affinity to warmer micro-climates (i.e. oyster ponds) (Boyer, Arzul & Bonnet 2012), but there is little hard data to substantiate this claim (Carrasco et al. 2008).

During experimental infection of *P. grani* with *M. refringens* and *M. maurini* (now *M. pararefringens*), differences in transmission efficacy between the two were found. The results showed that *M. pararefringens* did not proliferate in copepods after ingestion, while the opposite was true for *M. refringens* (Carrasco et al. 2008). Preliminary analysis of the relative abundance of different copepod groups in Agapollen did not detect *P. grani* (Appendix C), which makes the validity of this species being the intermediate host in the life cycle of *M. pararefringens* untenable. Nevertheless, a closely related species, *Acartia omorii*, was found in relatively high numbers. This species was introduced to Europe from the Pacific Ocean in 2005 (Seuront 2005), before the first reported instance of marteiliosis in 2016. However, if *A. omorii* does play a role in the life cycle of *M. pararefringens*, it does not explain the endemic presence of the parasite in the Mediterranean before the first reports of this copepod in Europe. *A. omorii* is closely related to the native *A. clausi*, which has been suggested as an intermediate host in *M. refringens* (Carrasco et al. 2007). Figure 24 shows a hypothetical life cycle of *M. pararefringens* if a copepod species is the intermediate host. Based on the presence of the parasite in *P. grani* gonads (Carrasco et al. 2008), the parasite could lay dormant in the resting eggs during winter and either infect the mussels by sporulation from the copepod or through ingestion of the copepod. Of course, this is conjecture and further research is necessary.

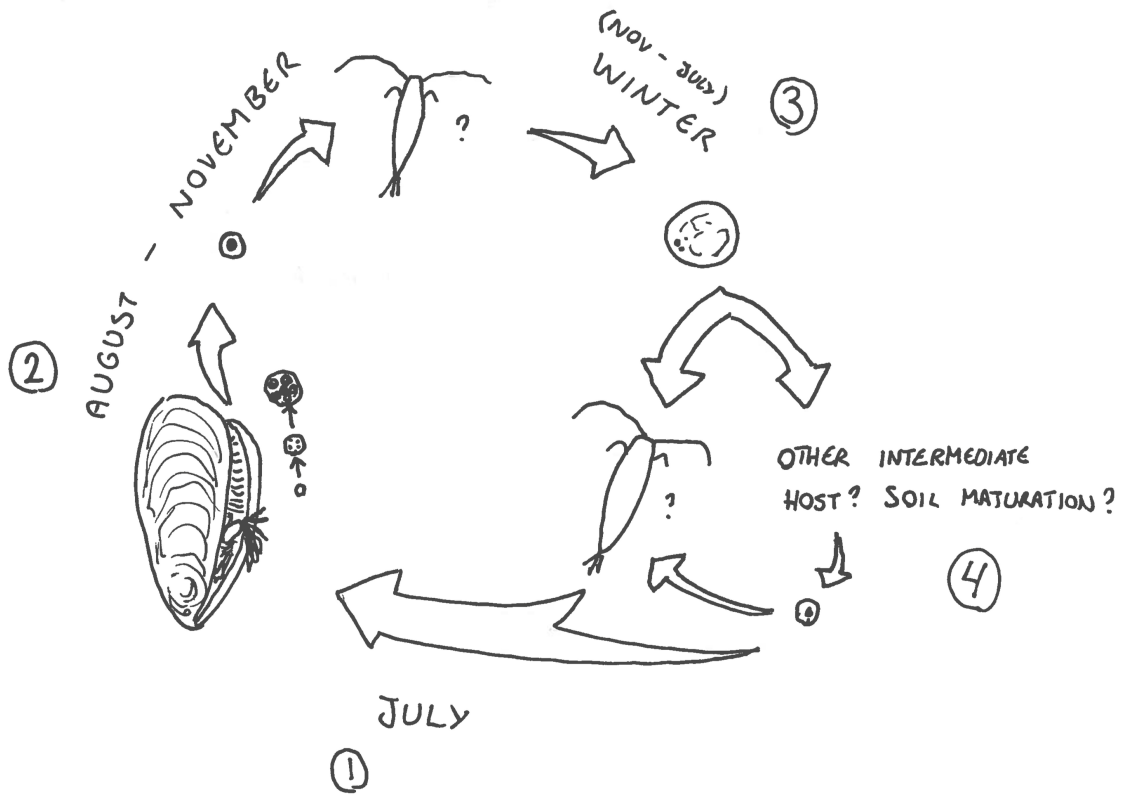

Figure 24: The potential life cycle of *Marteilia pararefringens*. Only the development of the parasite in *Mytilus edulis* is proven. (1) Transmission of the parasite occurs from July from an unknown intermediate host. (2) Development and sporulation of the parasite occurs from August to November. The spores infect some intermediate host, perhaps a copepod in which the parasite might migrate to the oocytes to infect the next generation of copepods through the resting eggs. (3) The parasite lays dormant during the winter as no new infections occur in the mussels. (4) If resting copepod eggs are infected, these could either develop inside copepods and sporulate, or the eggs could be ingested by other fauna and develop there. This stage is also unknown.

4.3 The origin of *M. pararefringens* in Norway

It is a matter of conjecture when discussing the potential transmission of *M. pararefringens* to other mussel populations in Norway, especially considering the limited information on its life cycle and origin. The question of where *M. pararefringens* came from is puzzling. The movement of molluscs between different sites is recognized as the greatest risk to the spread of disease (Isabelle Arzul 2018). Indeed, this practice has led to the introduction of devastating pathogens that have caused mass mortalities among native stocks, particularly in France and Spain. The first drastic mortalities due to the then-unknown parasite *M. refringens* were reported in oysters in 1967 (Comps 1970) (Renault 1996). By the time a diagnosis had been found, oyster had been transported to grow-out sites from the bay of Brest to Marennes (west coast of France) and in Galicia, Spain (Renault 1996). Abnormally high mortalities were reported from these locations in 1970, as well as other surround regions (Comps 1970). Oysters from diseased stocks were still being exported to other regions, including the

Netherlands. In this area, however, no abnormal mortalities were seen. In fact, the parasite did not seem to be able to propagate, even though the environmental parameters such as temperature and salinity are practically identical to France and Spain (Renault 1996). Even though sporulating stages were present in oysters after a few months in Dutch waters, no outbreaks were recorded. This evidence indicates that the parasite was not able to fulfil its life cycle, perhaps due to the absence of an intermediate host (van Banning 1979).

Considering the poor survivability of *Marteilia* spores in the environment (Wesche, Adlard & Lester 1999) and the Dutch 'barrier' hindering the natural spread of marteiliosis north, the presence of *M. pararefringens* in Norway and Sweden becomes even more confusing. The presence of a *Marteilia* species in Sweden must have its origins from importation of carriers from infected stocks (never been documented) or it may have spread from Denmark, through Kattegat and north along the coast. Surveillance efforts in Denmark have never confirmed the presence of *M. refringens* (O- or M-type), resulting in the discontinuation of the programme in 2016 for all regions except Nissum Bredning (the most western part of Limfjorden) (Danish Veterinary and Food Administration 2017). However, only oysters have been included in the surveillance program – mussels have never been monitored here, so the presence of *M. pararefringens* cannot be excluded.

If *M. pararefringens* reached Sweden through natural processes, how did the parasite reach Norwegian waters? The health status of oyster and mussel stocks have been consistently good, with no diseases being detected since 2009 (Mortensen et al. 2018), with two notable exceptions: *M. pararefringens* detected in Aga, Hordaland (Mortensen et al. 2017a) and a peculiar, unreported case of *Marteilia* sp. described by Aarab, Godal, & Bechmann (2011) in Førlandsfjorden, Rogaland. Whether the parasite reached Norway through natural spread or importation is not clear. Survivability of suspended *Marteilia* spores is poor, so long-distance spread is improbable (Wesche, Adlard & Lester 1999) unless contained within another species, such as a planktonic intermediate host. If natural spread is the cause, the parasite should be present all along the coast where the bivalve and intermediate host is present and can complete the life cycle, which has not been demonstrated. IMR performs samplings of oysters and mussels along the coast at only five locations, from Ytre Hvaler, Østfold to Aga, Bømlo, Hordaland. The limited sampling sites might not be able to detect infections in sporadic local populations of mussels. After the detection of *M. pararefringens* in Agapollen, surveillance efforts increased in the region, including Espevikpollen, Kuleseid,

Innerøypollen, and Kvalvågnes (Figure 4). Espevikpollen and Innerøypollen are old oyster ponds that have been prolific in the live movement of animals between sites. Kvalvågnes is an active grow-out site for shellfish that has received oyster spat from Agapollen. These sites were included because of their importance in the oyster-rearing network (NET-østers), where free movement of spat and adults between sites occurred frequently from the 1980s to 2010. Kuleseid is a wild oyster bed at Bømlo, sampled to see whether the parasite has spread to areas not associated with cultivation of bivalves.

The importation of molluscs into Norway is modest but has occurred. The last reported cases involved *C. gigas* in the 1980s deployed in Espevikpollen and Vallersund, Trøndelag. Through PCR analysis and verification using RFLP, two mussels from Espevikpollen were shown to be positive for the parasite. Whether *M. pararefringens* came with *C. gigas* imports is not clear, but the first cases of *M. refringens* occurred in France around the same time this oyster was imported to that region. In France, official reports say the oyster was imported in 1972 (Goulletquer & Heral 1997), five years *after* the first cases of *M. refringens* appeared (Comps 1970). However, the unofficial report is that farmers fascinated by the robustness of the oyster imported it in 1965-1966, one to two years *before* the first reports of the parasite appeared (Mortensen et al. 2017b, p. 181). *C. gigas* is not the type host for either *M. refringens* or *M. pararefringens*, but spores or infected intermediate hosts can be present in the water contained within the shell. When such 'infected' molluscs are transported from one area to another, they can release the disease into the water column and potentially infecting the naïve ecosystem. Whether Espevikpollen was infected by imported Pacific oysters is uncertain. As mentioned, only PCR analysis was performed on the mussels gathered here in July. Mussels from the oyster pond should be sampled for histology and PCR screening in autumn to study the parasite dynamics here.

How Agapollen became infected with the parasite is unknown but the surveillance program does provide an, albeit limited, time frame. The sampling of oysters bi-yearly since 2006 (n = 720) makes it statistically unlikely that even *cul de sac* infections would go undetected, which one could reasonably argue stems from a recent introduction of the parasite to the oyster pond. However, mussels histologically screened in 2016 were found to be infected by the parasite, while the oysters were negative. There were a few oysters that were PCR positive for the parasite, but no infection was detected by histology. The prevalence in the mussel population varies but is maintained throughout the year, which means the parasite

persists without the involvement of the oysters (IMR archive). It has been postulated that the spread of *Marteilia* sp. can occur through illegal introduction of infected molluscs from abroad or via ships containing infected molluscs on the hull or infected intermediate hosts in the ballast water (Virvilis & Angelidis 2006; Brenner et al. 2014). That being said, such hypotheses are usually associated with natural beds in close proximity to shipping lanes or in active aquaculture sites (Virvilis & Angelidis 2006), none of which is applicable to Agapollen, an idle site since 2010.

Agapollen used to be a prolific hatchery, transporting spat to grow-out sites in the region, as well as importing adult oysters for packaging. It is important here to note that the movement of mussels has not been documented in Norway, so disease spread by live movement must occur through oysters contaminated by spores or infected intermediate hosts. No Pacific oysters have been imported here, but movement of oysters between Agapollen and Espevikpollen has occurred. Perhaps *M. pararefringens* first infected mussels in Espevikpollen after importation of *C. gigas*, followed by propagation of the parasite in its type host. The passive filtration of spores or infected intermediate hosts could harbour in oysters moved to Agapollen, which could then have infected the mussel populations here.

Speculations on the parasite's origins and its diffusion in nearby regions is impossible to clarify. Nevertheless, it is important to understand the development and transmission of the parasite to hinder the spread into other areas where the impact could be massive. The continuation of the screening of mussels for *M. pararefringens* in Agapollen is important, but other oyster ponds and grow-out sites should also be monitored to study whether the movement of oysters has spread the disease to these areas. The peculiar case of *Marteilia* sp. reported by Aarab, Godal & Bechmann (2011) should also be studied further.

4.4 Evaluation of the experimental design

Deployment size and placement time

The period when the first group of mussels was deployed was based on the assumption that transmission occurs some time during spring/early summer. The cohabitation experiment was therefore started in May, with 250 healthy mussels. The second group also consisted of 250 mussels, deployed to see whether transmission could occur during sporulation from infected mussels in autumn. The sample size was chosen based on the assumption that 30 mussels

could be extracted every six weeks for one year. This would mean roughly 8 samplings of 30 mussels each –a total of 240 per group. Obviously, this does not leave sufficient margin of error to account for mortality or predation. The natural cumulative mortality varies in mussels, but is generally between 10% and 20%[a] (Myrand, Guderley & Himmelman 2000; McGrorty et al. 1990; Karayücel & Karayücel 1999). Based on this, one could expect 25-50 mussels per group perishing during the study. This does not take into account the relatively high mortality associated with the stress from handling, which is fairly common (Myrand, Guderley & Himmelman 2000; Karayücel & Karayücel 1999). It also does not take into account mortality associated with marteiliosis.

During sampling in August, numerous dead mussels were found in the two oyster baskets. What caused the high mortality is not certain, but the summer of 2018 was unusually hot and dry in Hordaland. Higher mortality rates of *M. edulis* have been associated with increased water temperatures, especially in late July and after major spawning events (Myrand, Guderley & Himmelman 2000). Several of the mussels from May and a few from July were mature, ready to spawn or recently spawned. It is reasonable that others from the same group were at a similar developmental stage, which could have reduced their tolerance to stress associated with increased temperatures or other factors, such as infection.

A possibility for the mortality could be a high infection intensity during the summer. The prevalence increased sharply between July and August, with a high intensity of infection (5) in a quarter of the sampled individuals (Figure 16). It is evident that the parasite matured rapidly from July (first positive PCR result) to August (first positive histology result), with advanced sporulation stages present. Considering the high mortality associated with *Marteilia* spp. throughout its geographic distribution, it is reasonable to believe that some of the mortality witnessed could be attributed to *M. pararefringens*. However, there have never been registered any significant mortalities of either mussels or oysters attributed to *M. pararefringens* recently discovered in Sweden, England, Ireland or Norway (Kerr et al. 2018; Laing et al. 2014). The second group, deployed in October, also consisted of 250 mussels. High mortalities were not reported here, which could support the infection-based mortality as these were never infected with *M. pararefringens*. However, winter mortalities are generally less intensive than those in the summer (McGrorty et al. 1990).

[a] Up to 40% if bird predation is accounted for, but this was not applicable for this experiment.

Screening

Screening ten microscope fields for each mussel was time-consuming, but important. However, the results could have been strengthened if the method would have been improved slightly. There are several studies that have reported preliminary infection in the gills and palps prior to infection of the digestive tract in *M. refringens* (Grizel 1979; Robledo & Figueras 1995). Though the palps were screened (if present), the gills were not studied as a potential infection site due to the work load this would present. *In situ* hybridization could have mediated the issue as it makes detection of the parasite easier, but it is labour intensive. There is also an issue with sequence similarity between certain *Marteilia* strains, such as *M. refringens* and *M. pararefringens* or *M. refringens* and *M. sydneyi* (Berthe et al. 2004). There does not yet exist *M. pararefringens*-specific primers for use in *in situ* hybridization, so positive histological results cannot diagnose of species level.

Temperature

The effect of temperature on *Marteilia* spp. development is well documented (Audemard et al. 2001; Berthe et al. 2004; Anestis et al. 2010; Murray et al. 2012; Carrasco, Green & Itoh 2015). 17°C seems to be the trigger for parasite multiplication and transmission in *M. refringens*, but this has not been documented in *M. pararefringens* (Audemard et al. 2001; Carrasco, Green & Itoh 2015). Considering the prolific development of the parasite in this study, it would have been interesting to compare the results found to the temperature in and around Agapollen.

qPCR vs Intensity score

It could have been useful to have analysed the mussels using quantitative PCR (qPCR) and comparing the Ct values with the PIDT/intensity scores. This could give an indication whether these two methods achieve comparable results. If so, it could greatly reduce the effort needed to identify the severity of the parasite infection as qPCR is vastly less labour-intensive compared to the screening method described by Villalba et al. (1993).

However, PCR analysis is prone to false negatives either due to sampling error (e.g. small abundance of pathogen DNA) or due to the presence of inhibitors that affect the DNA

polymerase. Such cases have been described (Aranguren & Figueras 2016) and require testing of different DNA extraction methods and qPCR kits.

Plankton samples

Plankton samples were of particular interest due to the potential role *P. grani* could have in the life cycle of *M. refringens* (Arzul et al. 2013; Boyer et al. 2013; Carrasco et al. 2008; Noèlia Carrasco et al. 2007; Audemard et al. 2002). However, in the absence of any systematic division into planktonic groups, the results are not useful in narrowing down which species is the likely intermediate host. The PCR results also do not give precedent to assume the plankton are infected because this must be validated by other techniques as well, such as histology or *in situ* hybridization (Burreson 2008). To improve the results gathered from the fauna samples, plankton should have been sorted into taxonomic groups before PCR analysis. This was not completed due to the work load associated with this process for the thesis. However, this is an interesting area of research and will be studied further at IMR.

5 Conclusions

Through the cohabitant trial at Aga using healthy mussels, the development of *M. pararefringens* in *M. edulis* could be described. The transmission time for *M. pararefringens* was found to be around July to August. Sporulation occurs from August to November, indicated by the presence of mature sporangia in the digestive tubules and lumina. No transmission of the parasite to healthy mussels occurred from October to March.

Transmission of the parasite from infected donor mussels to healthy mussels was unsuccessful, indicating the need for an intermediate host. Prevalence of the parasite has been shown in plankton samples from July to October, but the intermediate host has not been conclusively determined.

6 Future perspectives

To further pinpoint the transmission window and properly evaluate the progression of *M. pararefringens* in *M. edulis*, a new larger batch of naïve mussels should be deployed at the same period as described in this thesis. Sampling should be performed at shorter intervals, e.g. every two-four weeks, particularly during the summer and autumn months. The preliminary infection sites could not be described in this study due to the rapid development of the parasite from July to August. It is necessary to evaluate the presence of the parasite during the winter to identify infection sites and developmental stage present at this time.

Research into the copepod abundance in Agapollen through sediment and water samples throughout the year should commence in order to elucidate the complete life cycle of the parasite. Plankton should be grouped into taxa and PCR analysed for the presence of *M. pararefringens.* Any positive results must be verified using *in situ* hybridization (or some other technique) to determine whether the samples are infected or not. Promising finds should be researched further, including laboratory transmission trials to study whether the parasite can be transferred between hosts.

In order to maintain tight control over the potential spread of *M. pararefringens,* the surveillance programme at IMR should continue. The presence of the parasite has already been shown in Espevikpollen in July. The inclusion of more sites that have been in contact with the former network of oyster producers is important in order to identify whether the parasite has spread to other locations in Norway.

References

Aarab, N., Godal, B.F. & Bechmann, R.K. 2011, 'Seasonal variation of histopathological and histochemical markers of PAH exposure in blue mussel (*Mytilus edulis* L.)', *Marine Environmental Research*, vol. 71, no. 3, pp. 213–7.

Adlard, R.D. & Nolan, M.J. 2015, 'Elucidating the life cycle of *Marteilia sydneyi*, the aetiological agent of QX disease in the Sydney rock oyster (*Saccostrea glomerata*)', *International Journal for Parasitology*, vol. 45, no. 6, pp. 419–26.

Alderman, D.J. 1979, 'Epizootiology of *Marteilia refringens* in Europe.', *Mar. Fish. Rev.*, pp. 67–9.

Allam, B. & Raftos, D. 2015, 'Immune responses to infectious diseases in bivalves', *Journal of Invertebrate Pathology*, vol. 131, pp. 121–36.

Anestis, A., Pörtner, H.O., Karagiannis, D., Angelidis, P., Staikou, A. & Michaelidis, B. 2010, 'Response of *Mytilus galloprovincialis* (L.) to increasing seawater temperature and to marteliosis: Metabolic and physiological parameters', *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology*, vol. 156, no. 1, pp. 57–66.

Annabi-Trabelsi, N., Rebai, R.K., Ali, M., Subrahmanyam, M.N.V., Belmonte, G. & Ayadi, H. 2018, 'Egg production and hatching success of *Paracartia grani* (Copepoda, Calanoida, Acartiidae) in two hypersaline ponds of a Tunisian Solar Saltern', *Journal of Sea Research*, vol. 134, no. December, pp. 1–9.

Aranguren, R. & Figueras, A. 2016, 'Moving from histopathology to molecular tools in the diagnosis of molluscs diseases of concern under EU legislation', *Frontiers in Physiology*, vol. 7, no. NOV.

Arzul, I. 2018, 'Situation of European mollusc production regarding diseases', *Bulletin of the European Association of Fish Pathologists*, vol. 38, no. 3, pp. 130–9.

Arzul, Isabelle 2018, 'Situation of European mollusc production regarding diseases', *Bulletin of the European Association of Fish Pathologists*, vol. 38, no. 3, pp. 130–8.

Arzul, I., Chollet, B., Boyer, S., Bonnet, D., Gaillard, J., Baldi, Y., Robert, M., Joly, J.Â.P., Garcia, C. & Bouchoucha, M. 2013, 'Contribution to the understanding of the cycle of the protozoan parasite *Marteilia refringens*', *Parasitology*, vol. 141, no. 2, pp. 227–40.

Arzul, I., Corbeil, S., Morga, B. & Renault, T. 2017, 'Viruses infecting marine molluscs', *Journal of Invertebrate Pathology*, vol. 147, pp. 118–35.

Audemard, C., Barnaud, A., Collins, C.M., Le Roux, F., Sauriau, P.G., Coustau, C., Blachier, P. & Berthe, F.C.J. 2001, 'Claire ponds as an experimental model for *Marteilia*

refringens life-cycle studies: New perspectives', *Journal of Experimental Marine Biology and Ecology*, vol. 257, no. 1, pp. 87–108.

Audemard, C., Le Roux, F., Barnaud, A., Collins, C., Sautour, B., Sauriau, P.G., De Montaudouin, X., Coustau, C., Combes, C. & Berthe, F. 2002, 'Needle in a haystack: Involvement of the copepod *Paracartia grani* in the life-cycle of the oyster pathogen *Marteilia refringens*', *Parasitology*, vol. 124, no. 3, pp. 315–23.

Auffret, M. & Poder, M. 1983. 'Recherches sur *Marteilia maurini*, parasite de *Mytilus edulis* sur les côtes de Bretagne nord. *Revue des Traveaux de l'Institute de Pêches Maritimes,* vol. 47. pp. 106-109.

Balouet, G. 1979, '*Marteilia refringens* – Considerations of the life cycle and development of Aber disease in *Ostrea edulis.' Mar. Fish. Rev.,* vol. 41, pp. 64-66.

Balseiro, P., Montes, A., Ceschia, G., Gestal, C., Novoa, B. & Figueras, A. 2007, 'Molecular epizootiology of the European *Marteilia* spp., infecting mussels (*Mytilus galloprovincialis* and *M. edulis*) and oysters (*Ostrea edulis*): An update', *Bulletin of the European Association of Fish Pathologists*, vol. 27, no. 4, pp. 148–56.

van Banning, P. 1979, 'Haplosporidian diseases of imported oysters, *Ostrea edulis,* in Dutch estuaries', *Marine Fisheries Review*, vol. 41, no. 1–2, pp. 8–18.

Bass, D., Ward, G.M. & Burki, F. 2019, 'Ascetosporea', *Current Biology*, vol. 29, no. 1, pp. R7–8.

Berthe, F.C.J., Pernas, M., Zerabib, M., Haffner, P., Thébault, A. & Figueras, A.J. 1998, 'Experimental transmission of *Marteilia refringens* with special consideration of its life cycle', *Diseases of Aquatic Organisms*, vol. 34, no. 2, pp. 135–44.

Berthe, F.C.J., Le Roux, F., Adlard, R.D. & Figueras, A. 2004, 'Marteiliosis in molluscs: A review', *Aquatic Living Resources*, vol. 17, no. 4, pp. 433–48.

Bignell, J.P., Dodge, M.J., Feist, S.W., Lyons, B., Martin, P.D., Taylor, N.G.H., Stone, D., Travalent, L. & Stentiford, G.D. 2008, 'Mussel histopathology: Effects of season, disease and species', *Aquatic Biology*, vol. 2, no. 1, pp. 1–15.

Boyer, S., Arzul, I. & Bonnet, D. 2012, 'Some like it hot: *Paracartia grani* (Copepoda: Calanoida) arrival in the Thau lagoon (south of France - Mediterranean Sea)', *Marine Biodiversity Records*, vol. 5, no. September 2012, pp. 1–9.

Boyer, S. & Bonnet, D. 2013, 'Triggers for hatching of *Paracartia grani* (Copepoda: Calanoida) resting eggs: An experimental approach', *Journal of Plankton Research*, vol. 35, no. 3, pp. 668–76.

Boyer, S., Chollet, B., Bonnet, D. & Arzul, I. 2013, 'New evidence for the involvement of

Paracartia grani (Copepoda, Calanoida) in the life cycle of *Marteilia refringens* (Paramyxea)', *International Journal for Parasitology*, vol. 43, no. 14, pp. 1089–99.

Brenner, M., Fraser, D., Van Nieuwenhove, K., O'Beirn, F., Buck, B.H., Mazurié, J., Thorarinsdottir, G., Dolmer, P., Sanchez-Mata, A., Strand, O., Flimlin, G., Miossec, L. & Kamermans, P. 2014, 'Bivalve aquaculture transfers in Atlantic Europe. Part B: Environmental impacts of transfer activities', *Ocean and Coastal Management*, vol. 89, pp. 139–46.

Brooks, S.J. & Farmen, E. 2013, ' The Distribution of the Mussel *Mytilus* Species Along the Norwegian Coast ', *Journal of Shellfish Research*, vol. 32, no. 2, pp. 265–70.

Burreson, E.M. 2008, 'Misuse of PCR assay for diagnosis of mollusc protistan infections', *Diseases of Aquatic Organisms*, vol. 80, no. 1, pp. 81–3.

Butt, D. & Raftos, D. 2008, 'Phenoloxidase-associated cellular defence in the Sydney rock oyster, *Saccostrea glomerata*, provides resistance against QX disease infections', *Developmental and Comparative Immunology*, vol. 32, no. 3, pp. 299–306.

Carrasco, N., Arzul, I., Chollet, B., Robert, M., Joly, J.P., Furones, M.D. & Berthe, F.C.J. 2008, 'Comparative experimental infection of the copepod *Paracartia grani* with *Marteilia refringens* and *Marteilia maurini*', *Journal of Fish Diseases*, vol. 31, no. 7, pp. 497–504.

Carrasco, N., Green, T. & Itoh, N. 2015, '*Marteilia* spp. parasites in bivalves: A revision of recent studies', *Journal of Invertebrate Pathology*, vol. 131, pp. 43–57.

Carrasco, N., Hine, P.M., Durfort, M., Andree, K.B., Malchus, N., Lacuesta, B., González, M., Roque, A., Rodgers, C. & Furones, M.D. 2013, '*Marteilia cochillia* sp. nov., a new *Marteilia* species affecting the edible cockle *Cerastoderma edule* in European waters', *Aquaculture*, vol. 412–413, pp. 223–30.

Carrasco, N., López-Flores, I., Alcaraz, M., Furones, M.D., Berthe, F.C.J. & Arzul, I. 2007, 'Dynamics of the parasite *Marteilia refringens* (Paramyxea) in *Mytilus galloprovincialis* and zooplankton populations in Alfacs Bay (Catalonia, Spain)', *Parasitology*, vol. 134, no. 11, pp. 1541–50.

Carrasco, Noèlia, López-Flores, I., Alcaraz, M., Furones, M.D., Berthe, F.C.J. & Arzul, I. 2007, 'First record of a *Marteilia* parasite (Paramyxea) in zooplankton populations from a natural estuarine environment', *Aquaculture*, vol. 269, no. 1–4, pp. 63–70.

Comps, M., 1970. 'Observations sur les causes d'une mortalité anormale des huîtres plates (*Ostrea edulis* L.) dans le basin de Marenne. Rev. Trav. Inst. Pêches Marit. 34, pp. 317-326.

Comps, M. 1983, 'Morphological study of *Marteilia chirstienseni* sp. n. parasite of *Scrobicularia piperata* P. (mollusc pelecypod)', *Rev. Trav. Pêches marit.,* vol 47, pp. 99-104.

Comps, M., Grizel, H., Papayanni, Y., 1982. 'Infection parasitaire causée par *Marteilia maurini* sp. nov. chez la moule *Mytilus galloprovincialis*. *J. Cons. Int. Explor. Mer*. pp. 1-3.

Comps, M., Park, M.S., Desportes, I. 1986, 'Etudde ultrastructurale de *Marteilioides chungmuensis* n. g., n. sp. parasite des ovocytes de l'huitre *Crassostrea gigas*', *Th. Protistilogica,* vol. 22, pp. 279-285.

Dahms, H.U. 1995, 'Dormancy in the Copepoda - an overview', *Hydrobiologia*, vol. 306, no. 3, pp. 199–211.

Danish Veterinary and Food Administration 2017, *Animal Health in Denmark 2016*.

Deportes, I. & Perkins, F.O. 1990, 'Phylum Paramyxea' In: Margulis, L., Corliss, J.O., Melkonian, M., Chapman, D.I. (eds.) *Handbook of Protocista*, Jones and Bartlett, Boston, pp. 30-35

EFSA Panel 2007, 'Scientific opinion of the panel on animal health and welfare on possible vector species and live stages of susceptible species not transmitting disease as regards certain mollusc diseases', *The EFSA Journal*, vol. 597, pp. 1-116.

Elgharsalli, R., Aloui-Bejaoui, N., Salah, H., Chollet, B., Jolu, J.P., Rovert, M., Couraleau, Y., Arzul, I. 2013, 'Characterization of the protozoan parasite *Marteilia refringens* infecting dwarf oyster *Ostrea stentina* in Tunisis', *J. Invert. Pathol,* vol. 112 (2), pp. 175-183.

Eurostat 2016, *Agriculture, forestry and fishery statistics*, *Statistical books*, vol. 61.

FAO 2018, 'The State of World Fisheries and Aquaculture 2018 - Meeting the sustainable development goals, THE STATE OF THE WORLD series of the Food and Agriculture Organization of the United Nations*', Aquaculture*, vol. 35.

Feist, S.W., Hine, P.M., Bateman, K.S., Stentiford, G.D. & Longshaw, M. 2009, '*Paramarteilia canceri* sp. n. (Cercozoa) in the European edible crab (*Cancer pagurus*) with a proposal for the revision of the order Paramyxida Chatton, 1911.', *Folia parasitologica*, vol. 56, no. 2, pp. 73–85.

Figueras, A. & Montes, J. 1988, 'Aber disease of edible oysters caused by *Marteilia refringens*', *Am. Fish. Soc. Spec. Publ,* vol. 18, pp. 38-46.

Figueras, A. & Robledo, J.A.F. 1993, 'Does the *Marteilia* present in mussels (*Mytilus galloprovincialis*) infect flat oysters (*Ostrea edulis*) ?', *Bulletin of the European*

Association of Fish Pathologists, vol. 13, no. 3, pp. 97–9.

Figueras, A.J., Jardonb, F., Caldas, R. & Jardonb, C.F. 1991, 'Diseases and parasites of rafted mussels (*Mytilus galloprovincialis* Lmk): preleiminary results', *Investigaciones Marinas*, vol. 99, pp. 17–33.

Fuentes, J., López, J., Mosquera, E., Vázquez, J., Villalba, A. & Álvarez, G. 2002, 'Growth, mortality, pathological conditions and protein expression of *Mytilus edulis* and *M. galloprovincialis* crosses cultured in the Ría de Arousa (NW of Spain)', *Aquaculture*, vol. 213, no. 1–4, pp. 233–51.

Garcia, C. Arzul, I., Robert, M., Chollet, B., Joly, J.P., Miossec, L., Francois, C., 2009. 'Detection of atypical *Marteilia refringens* in mussels, *Mytilus edulis* in France. In: 101st Annual Meeting National Shellfisheries Association (NSA), Savannah, Georgia, March 22-26, 2009.

Goulletquer, P. & Heral, M. 1997, 'Marine Molluscan Production Trends in France: From Fisheries to Aquaculture', *In: C.L. MacKenzie, V.G. Burrell, A. Rosenfield, H. W. (Ed.), The history, present condition, and future of the Molluscan fisheries of North America and Europe. NOAA Technical Report NMFS 129, Department of Commerce, Seattle, Washington, 1997, 137-164. NO*, pp. 137–64.

Grizel, H. 1979, '*Marteilia refringens* and oyster disease. Recent observations', *Mar. Fish. Rev.*, vol. 41, pp. 38–44.

Grizel, H. 1985, 'Étude des récentes épizooties de l'huître plate (*Ostrea edulis* Linné) et leur impac sur l'ostréiculture bretonne.' *Thèse Doctorat es Sciences, Univ. Sciences et Techniques du Languedoc,* Montpellier.

Grizel, H., Comps, M., Bonami, J.R., Cousserans, F., Duthoit, J.L., Pennec, M., 1974. 'Epizooty of the common oyster *Ostrea edulis*, Part 1. Study of the agent of digestive gland disease in *Ostrea edulis* Linne.' *Sci. Pêche* 240-241, pp. 1-30.

Grizel, H., Héral M., 1991, 'Introduction into France of the Japanese oyster *Crassostrea gigas*', J. Cons. Internat. Explor. Mer. 47, pp. 399-403.

Hillis, D.M. & Dixon, M.T. 1991, 'Ribosomal DNA: molecular evolution and phylogenetic inference', *Quarterly Review of Biology*, vol. 66, pp. 411-453.

Hovgaard, P., Mortensen, S., Strand, Ø. 2001, 'Skjell – biologi og dyrking', 1. ed. *Kystnæringen Forlag & Bokklubb AS,* pp. 38-39.

Howard, D.H., Lewis, J.L., Keller, B.J. & Smith, C.S. 2004, 'Histological Techniques for Marine Bivalve Mollusks and Crustaceans', *NOAA Technical Memorandum NOS NCCOS 5*.

Inoue, K., Waite, J.H., Matsuoka, M., Odo, S. & Harayama, S. 1995, 'Interspecific variations in adhesive protein sequences of *Mytilus edulis, M. galloprovincialis*, and *M. trossulus*.', *The Biological bulletin*, vol. 189, no. 3, pp. 370–5.

Itoh, N., Yamamoto, T., Kang, H.S., Choi, K.S., Green, T.J., Carrasco, N., Awaji, M., Chow, S. 2014, 'A novel paramyxean parasite, *Marteilia granula* sp. nov (Cercozoa), from the digestive gland of Manila clam, *Ruditapes philippinarum,* in Japan', *Fish Pathol.*, vol. 49 (4), pp. 191-193.

Kang, H.-S., Itoh, N., Limpanont, Y., Lee, H.-M., Whang, I. & Choi, K.-S. 2019, 'A Novel Paramyxean Parasite, *Marteilia tapetis* sp. nov. (Cercozoa) Infecting the Digestive Gland of Manila clam *Ruditapes philippinarum* from the Southeast Coast of Korea', *Journal of Invertebrate Pathology*, vol. 163, no. March, pp. 86–93.

Karayücel, S. & Karayücel, I. 1999, 'Growth and Mortality of Mussels (*Mytilus edulis* L.) Reared in Lantern Nets in Loch Kishorn, Scotland', *Turkish Journal of Veterinary and Animal Sciences*, vol. 23, no. 5, pp. 397–402.

Kerr, R., Ward, G.M., Stentiford, G.D., Alfjorden, A., Mortensen, S., Bignell, J.P., Feist, S.W., Villalba, A., Carballal, M.J., Cao, A., Arzul, I., Ryder, D. & Bass, D. 2018, '*Marteilia refringens* and *Marteilia pararefringens* sp. nov. are distinct parasites of bivalves and have different European distributions', *Parasitology*, pp. 1–10.

Kleeman, S.N., Adlard, R.D. & Lester, R.J.G. 2002, 'Detection of the initial infective stages of the protozoan parasite *Marteilia sydneyi* in *Saccostrea glomerata* and their development through to sporogenesis', *International Journal for Parasitology*, vol. 32, no. 6, pp. 767–84.

Kuchel, R.P., Aladaileh, S., Birch, D., Vella, N. & Raftos, D.A. 2010, 'Phagocytosis of the protozoan parasite, *Marteilia sydneyi*, by Sydney rock oyster (*Saccostrea glomerata*) hemocytes', *Journal of Invertebrate Pathology*, vol. 104, no. 2, pp. 97–104.

Laing, I., Dunn, P., Peeler, E.J., Feist, S.W. & Longshaw, M. 2014, 'Epidemiology of *Bonamia* in the UK, 1982 to 2012', *Diseases of Aquatic Organisms*, vol. 110, no. 1–2, pp. 101–11.

Limpanont, Y. Kang, H.S., Hong, H.K., Jeung, H.D., Kim, B.K., Le, T.C., Kim, Y.O., Choi, K.S. 2013, 'Molecular and histological identification of *Marteilioides* infection in Suminoe oyster *Crassostrea ariakensis*, Manila clam *Ruditapes philippinarum* and Pacific oyster *Crassostrea gigas* on the south coast of Korea', *J. Invertebr. Pathol.,* vol. 114, pp. 277-284.

Longshaw, M., Feist, S.W., Matthews, R.A. & Figueras, A. 2001, 'Ultrastructural

characterisation of *Marteilia* species (Paramyxea) from *Ostrea edulis, Mytilus edulis* and *Mytilus galloprovincialis* in Europe', *Diseases of Aquatic Organisms*, vol. 44, no. 2, pp. 137–42.

López-Flores, I., de la Herrán, R., Garrido-Ramos, M., Navas, J., Ruiz-Rejón, C., & Ruiz-Rejón, M. 2004, 'The molecular diagnosis of *Marteilia refringens* and differentiation between *Marteilia* strains infecting oysters and mussels based on the rDNA IGS sequence', *Parasitology*, vol. 129, pp. 411-419.

López-Flores, I., Robles, F., Valencia, J.M., Grau, A., Villalba, A., de la Herrán, R., Garrido-Ramos, M.A., Ruiz-Rejón, C., Ruiz-Rejón, M., & Navas, J.I. 2008, 'Detection of *Marteilia refringens* using nested PCR and *in situ* hybridisation in *Chamelea gallina* from Balearic Islands (Spain)', *Diseases of Aquatic Organisms*, vol. 82, pp. 79-87.

Ludwig, J.A. & Reynolds, J.F. 1988 'Statistical Ecology. A primer on methods and computing'. Vol 1. *John Wiley & Sons*, New York

McGrorty, S., Clarke, R., Reading, C. & Goss-Custard, J. 1990, 'Population dynamics of the mussel *Mytilus edulis*: density changes and regulation of the population in the Exe estuary, Devon', *Marine Ecology Progress Series*, vol. 67, no. Holm, pp. 157–69.

de Montaudouin, X. & Sauriau P.-G. 2000, 'Contribution to a synopsis of marine species richness in the Pertius Charentais Sea with new insight in soft-bottom macrofauna of the Marennes-Oléron Bay.' *Cah. Biol. Marc.,* vol. 41, pp. 181-222.

Montes, J., Longa, M.A., Lama, A. & Guerra, A. 1998, 'Marteiliosis of Japanese oyster (*Crassostrea gigas*) reared in Galicia NW Spain', *Bulletin of the European Association of Fish Pathologists*, vol. 18, no. 4, pp. 124–6.

Mortensen, S., Hald, F., Ronold, A., Klakegg, Ø. 2017b, 'Østers! Fra råvare til servering – den norske østerdyrkingens historie', 1. ed. *Forlaget Vigmostad Bjørke*, Bergen. pp. 125-200.

Mortensen, S., Sælemyr, L., Skår, C.K., Bøgwald, M. & Jelmert, A. 2019, 'The surveillance and control programme for bonamiosis and marteiliosis in European flat oysters, *Ostrea edulis*, and blue mussels, *Mytilus* sp. in Norway in 2018', *Rapport fra Havforskningen*, vol. 25-2018.

Mortensen, S., Sælemyr, L., Skår, C.K. & Jelmert, A. 2016, 'The surveillance and control programme for bonamiosis and marteiliosis in European flat oysters, *Ostrea edulis*, and blue mussels, *Mytilus* sp. in Norway in 2015', *Rapport fra Havforskningen*, vol. 22-2015.

Mortensen, S., Sælemyr, L., Skår, C.K. & Jelmert, A. 2017a, 'The surveillance and control

programme for bonamiosis and marteiliosis in European flat oysters, *Ostrea edulis*, and blue mussels, *Mytilus* sp. in Norway in 2016', *Rapport Fra Havforskingen*, vol. 23–2016.

Mortensen, S., Sælemyr, L., Skår, C.K. & Jelmert, A. 2018, 'The surveillance and control programme for bonamiosis and marteiliosis in European flat oysters, *Ostrea edulis*, and the blue mussel, *Mytilus* sp. in Norway in 2017', *Rapport fra Havforskningen*, vol. 24-2017.

Mortensen, S., Strand, A., Bodvin, T., Alfjorden, A., Skar, C.K., Jelmert, A., Aspán, A., Sælemyr, L., Naustvoll, L.J. & Albretsen, J. 2016, 'Summer mortalities and detection of ostreid herpesvirus microvariant in Pacific oyster *Crassostrea gigas* in Sweden and Norway', *Diseases of Aquatic Organisms*, vol. 117, no. 3, pp. 171–6.

Moyer, M.A., Blake, N.J., Arnold, W.S., 1993, 'An ascetosporan disease causing mass mortality in the Atlantic calico scallop, *argopecten gibbus* (Linnaeus, 1758)', *J. Shellfish Res.,* vol. 12 (2), pp. 305-310.

Murray, A.G., Marcos-Lopez, M., Collet, B. & Munro, L.A. 2012, 'A review of the risk posed to Scottish mollusc aquaculture from *Bonamia, Marteilia* and oyster herpesvirus', *Aquaculture*, vol. 370–371, pp. 7–13.

Myrand, B., Guderley, H. & Himmelman, J.H. 2000, 'Reproduction and summer mortality of blue mussels *Mytilus edulis* in the Magdalen Islands, southern Gulf of St. Lawrence', *Marine Ecology Progress Series*, vol. 197, pp. 193–207.

Novoa, B., Posada, D., & Figueras, A. 2005, 'Polymorphisms in the sequences of *Marteilia* internal transcribed spacer region of the ribosomal RNA genes (ITS1) in Spain: genetic types are not related with bivalve hosts', *Journal of Fish Diseases*, vol. 28, pp. 331-338.

Page, R.D.M. & Holmes, E.C. 1998, 'Molecular Evolution: A Phylogenetic Approach', *Blackwell Science, Londong (United Kingdom)*. ISBN 0-86542-889-1.

Pasqual, S., Villalba, A., Abollo, E., Garci, M., Gónzalez, A.F., Nombela, M., Posada, D., Guerra, A., 2010, 'The mussel *Xenostrobus securis:* a well-established alien invader in the Ria de Vigo (Spain, NE Atlantic)', *Biol. Invasions,* vol. 12 (7), pp. 2091-2103.

Perkins, F.O. 1976. 'Ultrastructure of sporulation in the European flat oyster pathogen *Marteilia refringens* – taxonomic implications', *J. Protozool,* vol. 23, pp. 64-74.

Perkins, F.O. & Wolf, P.H. 1976, 'Fine structure of *Marteilia sydneyi* sp. n.-haplosporidian pathogen of Australian oysters', *J. Parasitol.,* vol. 62, pp. 528-538.

Perrigault, M., Tanguy, A. & Allam, B. 2009, 'Identification and expression of differentially expressed genes in the hard clam, *Mercenaria mercenaria*, in response to quahog

parasite unknown (QPX)', *BMC Genomics*, vol. 10, p. 377.

Renault, T. 1996, 'Appearance and spread of diseases among bivalve molluscs in the northern hemisphere in relation to international trade', *Revue scientifique et technique (International Office of Epizootics)*, vol. 15, no. 2, pp. 551–61.

Robledo, J.A.F. & Figueras, A. 1995, 'The Effects of Culture-Site, Depth, Season, and Stock Source on the Prevalence of *Marteilia refringens* in Cultured Mussels (*Mytilus galloprovincialis* LMK.) from Galicia, Spain', *The Journal of Parasitology*, vol. 81, no. 3, p. 354.

Roch, P. 1999, 'Defense mechanisms and disease prevention in farmed marine invertebrates', *Aquaculture*, vol. 172, no. 1–2, pp. 125–45.

Roubal, F.R., Masel, J., Lester, R.J.G. 1989, 'Studies on *Marteilia sydneyi*, agent of QX disease in the Sydney rock oyster, *Saccostrea commercialis,* with implications for its life cycle.' *Aus. J. Mar. Freshwater Res.,* vol. 40, pp. 155-167.

Le Roux, F., Audemard, C., Barnaud, A., & Berthe, F.C.J. 1999, 'DNA probes as potential tools for the detection of *Marteilia refringens', Marine Biotechnology,* vol. 1, pp. 588-597.

Le Roux, F., Lorenzo, G., Peyret, P., Audemard, C., Figueras, a, Vivarès, C., Gouy, M. & Berthe, F. 2001, 'Molecular evidence for the existence of two species of *Marteilia* in Europe.', *The Journal of eukaryotic microbiology*, vol. 48, no. 4, pp. 449–54.

Ruiz, M., López, C., Lee, R.S., Rodríguez, R. & Darriba, S. 2016, 'A novel paramyxean parasite, *Marteilia octospora* n. sp. (Cercozoa) infecting the Grooved Razor Shell clam Solen marginatus from Galicia (NW Spain)', *Journal of Invertebrate Pathology*, vol. 135, pp. 34–42.

Segarra, A., Pépin, J.F., Arzul, I., Morga, B., Faury, N. & Renault, T. 2010, 'Detection and description of a particular Ostreid herpesvirus 1 genotype associated with massive mortality outbreaks of Pacific oysters, *Crassostrea gigas*, in France in 2008', *Virus Research*, vol. 153, no. 1, pp. 92–9.

Seuront, L. 2005, 'First record of the calanoid copepod *Acartia omorii* (Copepoda: Calanoida: Acartiidae) in the southern bight of the North Sea', *Journal of Plankton Research*, vol. 27, no. 12, pp. 1301–6.

Thrush, M.A., Pearce, F.M., Gubbins, M.J., Oidtmann, B.C. & Peeler, E.J. 2017, 'A Simple Model to Rank Shellfish Farming Areas Based on the Risk of Disease Introduction and Spread', *Transboundary and Emerging Diseases*, vol. 64, no. 4, pp. 1200–9.

Tiscar, P.G. & Mosca, F. 2004, 'Defense Mechanisms in Farmed Marine Molluscs',

Veterinary Research Communications, vol. 28, pp. 57–62.

Villalba, A., Mourelle, S.G., Lopez, M.C. & Carballal, M.J. 1993, 'Marteiliasis affecting cultured mussels *Mytilus galloprovincialis* of Glacia (NW Spain). I Etiology, phases of the infection, and temporal and spatial variablity in prevalence', *Diseases of Aquatic Organisms*, vol. 16, no. Table 1, pp. 61–72.

Virvilis, C. & Angelidis, P. 2006, 'Presence of the parasite *Marteilia* sp. in the flat oyster (*Ostrea edulis* L) in Greece', *Aquaculture*, vol. 259, no. 1–4, pp. 1–5.

Wesche, S.J., Adlard, R.D. & Lester, R.J.G. 1999, 'Survival of spores of the oyster pathogen *Marteilia sydneyi* (Protozoa, Paramyxea) as assessed using fluorogenic dyes', *Diseases of Aquatic Organisms*, vol. 36, no. 3, pp. 221–6.

Zrnčić, S., Le Roux, F., Oraić, D., Šoštarić, B. & Berthe, F.C.J. 2001, 'First record of *Marteilia* sp. in mussels *Mytilus galloprovincialis* in Croatia', *Diseases of Aquatic Organisms*, vol. 44, no. 2, pp. 143–8.

Appendix

6.1 Appendix A – Histology protocol

MOLLUSCS PROCESSING FOR DIAGNOSIS BY HISTOLOGY

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Editions

Edition	Date	Updated part
# 1	26/09/2006	Creation
# 2	08/10/2006	Modification of § 2 & 6.6
# 3	26/05/2011	Creation of § 6.4, modification of § 5.1, 6.5 and 7

MOLLUSCS PROCESSING FOR DIAGNOSIS BY HISTOLOGY

1. Scope

This procedure explains the techniques used for histological processing of common bivalves and abalones. It explains the processes of dissection, fixing, embedding and cutting tissue with a microtome before staining slides for histological examination.

2. References

- OIE. Manual of Diagnostic Tests for Aquatic Animals, current edition, Paris, France.
- Howard D.H., Lewis J.L., Keller B.J. & Smith C.S. (2004). Histological Techniques for Marine Bivalve Mollusks and Crustaceans, NOAA Technical Memorandum NOS NCCOS 5, 218 p.

3. General information

Tissue fixation preserves cellular details for examination by microscopy. An ideal fixative quickly penetrates tissue to prevent post-mortem damaging. It coagulates cell proteins by binding them together and hardens tissue to allow further histological processing (dehydration, embedding in paraffin and cutting with a microtome) without changing too much the shape of each organ. Embedding is the process of placing tissue in a firm medium to keep it intact when cutting sections with a microtome for histological examination

4. Equipment and environment

4.1. Equipment

- Scalpel or knife
- Razor blades (or used microtome blades)
- Gloves
- Tweezers
- Paper towelling
- Cassettes for histology
- Measuring cylinders
- Pots for tissue fixation
- Oven (42°C)to dry slides
- Histological slides
- Racks for histological slides
- Automatic tissue processor
- Embedding centre
- Cooling unit
- Metallic molds
- Microtome
- Heated waterbath
- Needle or paintbrush

4.2. Environment

- Well ventilated laboratory
- Fume hood

5. Preparation of fixatives

5.1. Reagents

- Ethanol 100%
- Ethanol 95%
- Ethanol 70%
- Xylene
- Paraffin
- Formaldehyde 36-40 %
- Filtered sea water
- Glycerin
- Acetic acid 99-100%
- Sodium dihydrogenophosphate (NaH_2PO_4, $2H_2O$)
- Distilled water
- Sodium hydroxyde pellets (NaOH)

5.2. Formulas for histology fixatives

Fixatives must be prepared under a fume hood.
Davidson's fixative can be used in routine survey. Formalin 10% in sea water is a general fixative, easily made and particularly interesting in the field or if travelling because of its simplicity.
Carson's fixative can be used for histology and allows also subsequent post-fixation with glutaraldehyde and osmium tetroxyde if transmission electronic microscopy (TEM) is needed for further investigation.

5.2.1. Davidson's fixative

Stock solution:

- Filtered sea water…….…….. 1200 ml
- Ethanol 95 % …….……. 1200 ml
- Formaldehyde 36-40 % …….. 800 ml
- Glycerin ……. 400 ml

Working solution:

- Stock solution ……..9 parts
- Glacial acetic acid……. 1 part (*add extemporaneously i.e. just prior to utilisation*)

5.2.2. Formalin 10% fixative

- Filtered sea water…….…….. 900ml
- Formaldehyde 36-40% …… 100ml
  pH neutral

5.2.3. Carson's fixative

- Dissolve in 900 ml of distilled water:
- Sodium dihydrogenophosphate ….....… 23.8 g
- Sodium hydroxide …….....… 5.2 g
- Then add:
- Formalin 36-40% ……..... 100 ml
  Mix thoroughly

6. Procedure

6.1. Preparing molluscs for histology

Open molluscs and quickly cut the adductor muscle(s) as close to the shell as possible (see the SOP "Opening bivalves"). Look for any clinical signs that can be observed on the shell (blister, boring sponge, brown ring, malformation, mud worm tunnel, pearl, pustules, scar) or on the soft parts (abscess, abnormal pigmentation, gill erosion, pustule, watery condition). Gently remove the body from the shell and put it on a paper towelling prior to slicing. Parts of abnormal tissue can be cut and fixed separately, for example in glutaraldehyde for Transmission Electron Microscopy (TEM).

6.2. Slicing molluscs before histology process

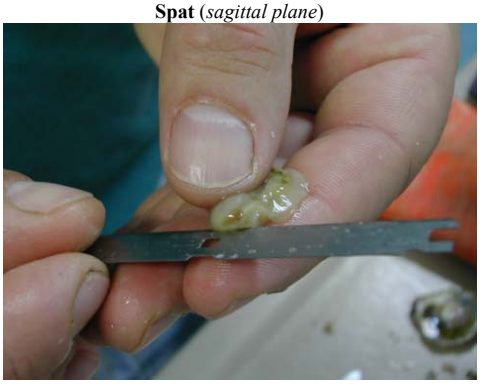
6.2.1. General information

Many molluscs from the same family share the same organisation. The general slicing process for the families of oysters, mussels, clams (or cockles), scallops and abalones which represent most of the molluscs produced in Europe is described. Very small molluscs (up to 2 cm long) can be fixed entirely.
Each proposed slicing plan is made to include most of the organs like digestive gland, gonad, intestine, gills, kidney.

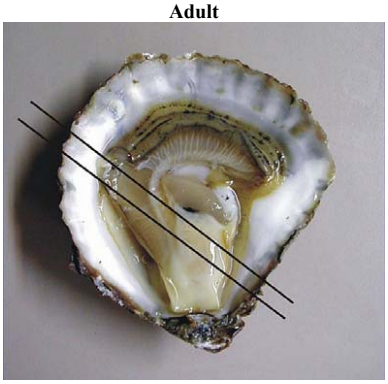
6.2.2. Oysters

Slice must be made as following:

Spat (*sagittal plane*)

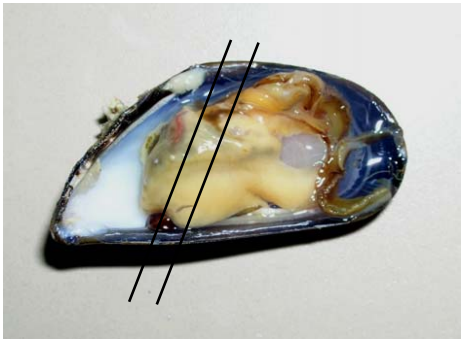


Adult



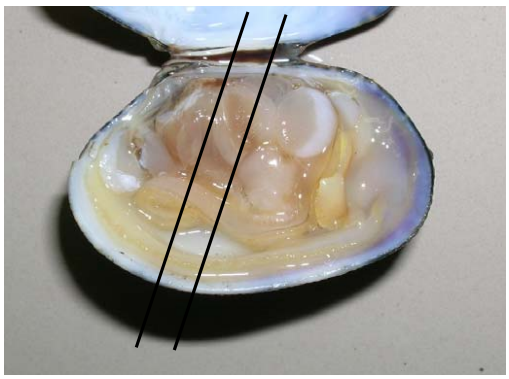
6.2.3. Mussels

Slice must be made as following (for **spat**, cut mussels by the saggital plane):



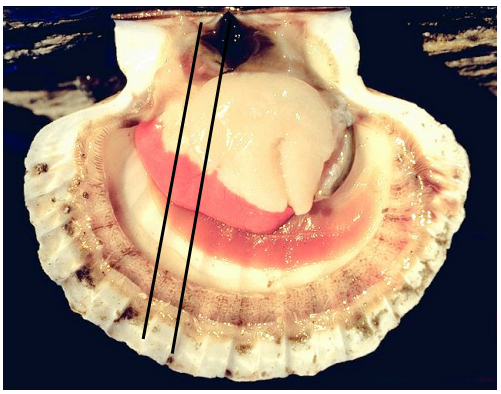
6.2.4. Clams

Slice must be made as following (for **spat**, cut clams by the saggital plane):



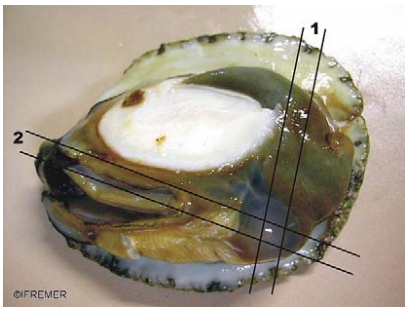
6.2.5. Scallops

Slice must be made as following (young and adult):



6.2.6. Abalones

2 slices must be made as following:



6.3. Fixation

Tissue fixation preserves cellular detail for examination by microscopy. Many fixatives are used and each one has its own properties for preserving such or such parts of the cell. OIE recommends the use of Davidson's fixative for general molluscan pathology. If *in situ* hybridation (ISH) is planned after histology (for confirmatory diagnosis for example) do not let the fixative process exceed 48 hours for better results; otherwise acetic acid would interfere with DNA preservation. Another possibility is to use Davidson's fixative without the acetic acid (i.e. use only the stock solution). Other fixatives can be used (see Howard et al., 2004, for more information).
Put slices of tissue of no more than 5 mm thickness in cassettes. Cassettes must be identified with the code of the sample and the number of the individual. Try to carefully set the tissue in the cassette so that each cut organ can be visible. If needed you can put other parts of the body in the cassette. Work must be done under a fume hood.

1. Put the slice of tissue in the cassette and orient it carefully
2. Immerge the cassette in the fixative (around 10 volumes of fixative for 1 volume of tissue)
3. Fixation should last 24 h minimum

6.4. Storage

If you need to keep fixed tissue for several days or weeks before further processing, transfer fixed tissue into **70% alcohol**.

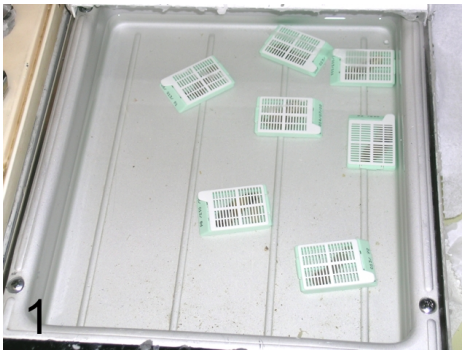
6.5. Tissue dehydration and infiltration

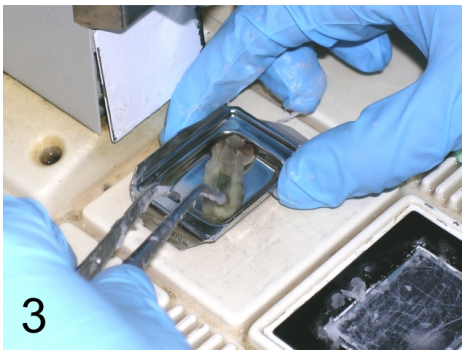
Once samples are fixed, they must be dehydrated and infiltrated with paraffin. This can be done manually or automatically by using an automatic tissue processor.
Here is an example of dehydration and infiltration program (process time can vary with the thickness and size of tissue):

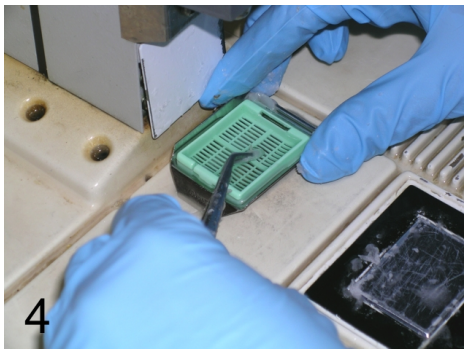
1. Ethanol 70% (30 minutes)
2. Ethanol 95 % (30 minutes)
3. Ethanol 95 % (30 minutes)
4. Ethanol 100 % (15 minutes)
5. Ethanol 100 % (30 minutes)
6. Ethanol 100 % (60 minutes)
7. Xylene (30 minutes)
8. Xylene (60 minutes)
9. Xylene (60 minutes)
10. Paraffin (45 minutes)
11. Paraffin (45 minutes)
12. Paraffin (45 minutes)
13. Paraffin (waiting bath before embedding)

6.6. Embedding

Embedding is the process of placing tissue in a block of paraffin to allow cutting sections with a microtome. Embedding centres are available with built-in paraffin baths and cooling units.
Remove tissue cassettes from the tissue processor and put them in the heated paraffin bath (picture 1). Take one cassette, place it on the heated surface and open it (picture 2). Put some paraffin in a mould (1/4 maximum). Take tissue from the cassette with heated forceps and orient it in the mould without trapping air bubbles (picture 3). Put the cassette on top of tissue (picture 4) and fill the mould with heated paraffin. Place the mould onto the cooling unit of the embedding centre. When the paraffin block has cooled, remove it from the mould for trimming and sectioning.







6.7. Sectioning

Good sectioning requires training and experience for the technician as well as a properly prepared material (i.e. well fixed and preserved and well dehydrated and embedded tissue). It is recommended that paraffin blocks be rough cut at room temperature and then precooled at 4 to 5°C (stored overnight in a fridge for example) before sectioning.

1. Rough cut blocks (using old blades for example)
2. Precool the paraffin blocks in a fridge or on a cooling table
3. Trim block until tissue is fully exposed
4. Set the microtome to 2-3 µm for section thickness
5. Cut ribbon of paraffin sections with the microtome
6. Put the ribbon on the heated waterbath (change water everyday)
7. Separate the sections
8. Dip coded slides under the tissue section and raised the slide from the water (guiding the section with a needle or brush)
9. Place the slides vertically in a rack to drain excess water
10. Dry in an oven or at room temperature
    Slides can be stored vertically on a rack or in a staining holder before staining process.

7. Safety information

Many hazardous chemicals are used during the histological process. All of them come in containers with special labels identifying their hazard characteristics such as **flammable**, **corrosive**, **reactive**, **toxic**, etc. Information on MSDS (material safety data sheets) can be found on Internet (for example: http://www.chemexper.com/). The **flash point** of a flammable product is the lowest temperature at which it can form an ignitable mix with air. Note that some paraffin media contain DMSO (dimethylsulfoxyd) which is slightly toxic: use of protective gloves is recommended.

Absolute Ethanol *(use under a fume hood)*
Eye: Causes severe eye irritation.
Skin: Causes moderate skin irritation.
Ingestion: May cause gastrointestinal irritation with nausea, vomiting and diarrhea.
Inhalation: Vapours may cause dizziness or suffocation.


Flash point: 16.6°C

H225 - Highly flammable liquid and vapor

Xylene *(use under a fume hood)*
Eye: Causes severe eye irritation .
Skin: Exposure may cause irritation. Prolonged contact may cause dermatitis.
Ingestion: May cause central nervous system depression, kidney damage and liver damage.
Inhalation: High concentrations may cause central nervous system effects characterised by nausea, headache, dizziness, unconsciousness and coma. Vapours may cause respiratory tract irritation. Irritation may lead to chemical pneumonitis and pulmonary oedema.


Flash point: 32°C

H332 - Harmful if inhaled
H315 - Causes skin irritation
H312 - Harmful in contact with skin
H226 - Flammable liquid and vapor

Formaldehyde *(use under a fume hood)*
Eye: Causes irritation. May result in cornea injury.
Skin: Causes skin irritation. Harmful if absorbed through the skin.
Ingestion: Causes gastrointestinal irritation with nausea, vomiting and diarrhea. May be harmful if swallowed.
Inhalation: Harmful if inhaled. Causes respiratory tract irritation.
Mutagenic effects have occurred in humans.


Flash point: 50°C

H314 - Causes severe skin burns and eye damage
H317 - May cause an allergic skin reaction
H370 - Causes damage to organs
H311 - Toxic in contact with skin
H331 - Toxic if inhaled
H301 - Toxic if swallowed
H351 - Suspected of causing cancer
H226 - Flammable liquid and vapor

Glacial acetic acid *(use under a fume hood)*
Eye: causes severe eye burns (with liquid or vapour)
Skin: May cause skin sensitisation. Causes severe burns with delayed tissue destruction.
Ingestion: May cause severe and permanent damage to the digestive tract.
Inhalation: May cause respiratory tract irritation with burning pain in the nose and throat, coughing, wheezing, shortness of breath and pulmonary oedema.


Flash point: 40°C

H314 - Causes severe skin burns and eye damage
H226 - Flammable liquid and vapor

Sodium hydroxide *(use under a fume hood when in solution)*
Eye: May cause eye irritation.
Skin: May cause skin irritation.
Ingestion: May cause gastrointestinal irritation with nausea, vomiting and diarrhea.
Inhalation: May cause respiratory tract irritation

H318 - Causes serious eye damage
H314 - Causes severe skin burns and eye damage

6.2 Appendix B – PCR and RFLP protocol

Ifremer Genetic and Pathology Laboratory, La Tremblade, France

Marteilia refringens detection and characterization by Polymerase Chain Reaction - Restriction Fragment Length Polymorphism

According to Le Roux et al. (2001)

1. Scope

This procedure explains a standard diagnostic test used for *Marteilia refringens* detection and characterization in flat oysters (e.g. *Ostrea edulis*) and mussels (*Mytilus edulis* and *M. galloprovincialis*) to confirm a previous histological or cytological diagnosis at the genus level. It allows a specific diagnosis between *Marteilia refringens* type O and *Marteilia refringens* type M.

2. References

Le Roux F., Lorenzo G., Peyret P., Audemard C., Figueras A., Vivarès C., Gouy M. & Berthe F., 2001. Molecular evidence for the existence of two species of Marteilia in Europe. *J. Eukaryot. Microbiol.* **48**, 4: 449-454.

OIE (2009). Manual of Diagnostic Tests for Aquatic Animals, section 2.4, Paris, France, (*web format of Manual of Diagnostic Tests*: http://www.oie.int/en/international-standard-setting/aquatic-manual/access-online)

3. Equipment and environmental conditions

This test requires the equipment and environmental conditions classically used for PCR assays:

- A closed hood equipped with an UV producing system to eliminate potential contaminations when preparing PCR mix
- Two complete sets of pipettes (2 µl; 20 µl; 200 µl and 1000 µl), the first one for DNA extraction, and the second one for PCR mix preparation.
- Three different pipettes: one pipette (2 µl) to dispense samples in PCR mix, one pipette (20µl) for BET sampling and another pipette (20 µl) to load PCR products in agarose gels
- Filter pipette tips (2 µl; 20 µl; 200 µl and 1000 µl) for DNA extraction, PCR mix preparation and sample dispensing
- Pipette tips (20 µl) to collect BET and to load amplification products in agarose gel
- A thermal cycler to perform amplifications
- A horizontal electrophoresis system for PCR products electrophoresis
- An UV table to observe PCR products after agarose gel electrophoresis
- A system to acquire pictures of the gels

Manipulator must wear a lab coat and gloves during all the different steps described bellow. Lab coat and gloves must be changed preferably after each main step: DNA extraction, preparation of PCR mix, sample dispensing, amplification and gel loading.

It is recommended to perform these different steps in different rooms. Amplification and gel loading / electrophoresis should particularly take place in a room separate from DNA extraction, PCR mix preparation and DNA dispensing.

4. Procedure

4.1. Sample preparation

DNA is extracted from a piece of digestive gland using QIAamp® DNA Mini Kit (QIAGEN) and following instructions for Tissue Protocol.

➔ **Carefully read the protocol given with the kit before starting DNA extraction**

1. Cut up to 25 mg of tissue into small pieces, place in a 1,5 ml microcentrifuge tube and add 180 µl of Buffer ATL
2. Add 20 µl Proteinase K, mix by vortexing and incubate at 56°C until the tissue is completely lysed (overnight). Vortex occasionally during incubation to disperse sample. Briefly centrifuge the 1,5 ml microcentrifuge tube to remove drops from the lid.
3. Add 200 µl Buffer AL to the sample, mix by pulse-vortexing for 15 s and incubate at 70°C for 10 minutes. Briefly centrifuge the 1,5 ml microcentrifuge tube to remove drops from the lid
4. Add 200 µl ethanol (96-100%) to the sample, and mix by pulse-vortexing for 15 s. Briefly centrifuge the 1,5 ml microcentrifuge tube to remove drops from the lid
5. Carefully apply the mixture from step 4 to the QIAamp Spin Column (in a 2 ml collection tube) without wetting the rim. Close the cap and centrifuge at 10 000 rpm for 1 min. Place the QIAamp Spin Column in a clean 2 ml collection tube (provided in the kit) and discard the tube containing the filtrate.
6. Carefully open the QIAamp Spin Column and add 500 µl Buffer AW1 without wetting the rim. Close the cap and centrifuge at 10 000 rpm for 1 min. Place the QIAamp Spin Column in a clean 2 ml collection tube (provided in the kit) and discard the collection tube containing the filtrate.
7. Carefully open the QIAamp Spin Column and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (14 000 rpm) for 3 min.
8. Place the QIAamp Spin Column in a new 2 ml collection tube (not provided in the kit) and discard the collection tube containing the filtrate. Centrifuge at full speed (14 000 rpm) for 1 min.
9. Place the QIAamp Spin Column in a clean 1,5 ml microcentrifuge tube (not provided in the kit) and discard the collection tube containing the filtrate. Carefully open the QIAamp Spin Column and add 50 µl of distilled water. Incubate 1 minute at room temperature and centrifuge at 10 000 rpm for 1 min.
10. Control the quality and efficacy of the extraction (for example by measuring the absorbance at 260 nm with a spectrophotometer or after electrophoresis in agarose gel).
11. Prepare dilutions of your samples in order to have a final DNA concentration of 100 ng/µl
12. DNA solutions are kept at 4°C until PCR analyses are performed

4.2. Polymerase Chain Reaction (PCR)

4.2.1. Reactives

- 10 X Buffer (provided with the Taq DNA Polymerase)
- $MgCl_2$ (provided with the DNA polymerase) (25 mM)
- Taq DNA Polymerase (Goldstar, Eurogentec) 5 U/µl
- dNTP Master Mix (20mM) must be diluted 10 fold (at 2mM) before use
- H_20 (free of DNA and RNA)

4.2.2. Primers

M2A 5'- CCG CAC ACG TTC TTC ACT CC - 3'
M3AS 5'- CTC GCG AGT TTC GAC AGA CG - 3'

(*corresponding to primers Pr4 and Pr5 in Le Roux* et al. *2001*)

4.2.3. PCR Mix

PCR mix for each tube is:

	Volume per tube	Final concentration
Buffer (10X)	5 µl	1X
$MgCl_2$ (25 mM)	5 µl	2,5 mM
dNTP (2mM)	5 µl	0,2 mM
M2A (100µM)	0,5 µl	1 µM
M3AS(100µM)	0,5 µl	1 µM
Taq polymérase (5U/µl)	0,5 µl	2,5 U
dH_2O	32,5 µl	

- 49 µl of this PCR mix is dispensed in each PCR tube
- 1 µl of extracted DNA (100 ng/µl) is added to each tube

Two types of control are used:

- **Negative** controls consist of dH_2O (1 µl for 49 µl of PCR Mix). They aim at detecting potential reactive contamination or working environment. One negative control should be included every 10 samples or after each batch of samples.
- **Positive** controls consist of DNA extracted from known highly infected oysters or mussels. They aim at checking the efficacy of the PCR reaction. One positive control should be included for each PCR analysis.

4.2.4. Amplification

Amplification cycles are performed in a thermal cycle apparatus (PTC-100 MJ Research, Inc.Perkin).

- Initial denaturation: 10 min at 94°C
- Amplification: 30 cycles (1 min at 94°C, 1 min at 55°C and 1 min at 72°C)
- Final elongation: 10 min at 72°C

4.2.5. Interpretation

A positive result is an amplicon of the appropriate size (412 bp) with all negative controls negative and all positive controls positive.

4.3. Restriction Fragment Length Polymorphism (RFLP)

4.3.1. Reactives

- 10 X Buffer (provided with the restriction enzyme)
- H_20 (free of DNA and RNA)
- *Hha*I (10 U/µl)

4.3.2. Digestion mix

Digestion mix for each tube is:

- 2 µl of the appropriate buffer
- 1 µl of enzyme
- 7 µl of dH_2O

↳ 10 µl of this digestion mix is dispensed in each tube
↳ 10 µl of PCR products are added to each tube

4.3.3. Digestion

Digestion is then performed by incubating samples for 1 hour at the temperature indicated by the manufacturer.

4.3.4. Interpretation

M2A / M3AS PCR products will be digested differently according to the type of *Marteilia refringens*.
Table below indicates expected restriction profiles:

	***Hha*I**
Marteilia refringens type M	157 bp + 156 bp + 68 bp + 31 bp
Marteilia refringens type O	226 bp + 156 bp + 31 bp

4.4. Electrophoresis

4.4.1. Reactives

- 50 X TAE (can be bought directly ready for use):
  - Tris base (40 mM) 242 g
  - glacial acetic acid (40 mM) 57,1 ml
  - $Na_2EDTA.2H_2O$ (1 mM) 18,61 g
  - dH_2O for 1 liter

  ↳ Ajust at pH 8
- Agarose gel:
  - 1 % for PCR products or 2% for RFLP products of agarose in 1X TAE

  ↳ Ethidium bromide (0,5 µg/ml) is added after cooling the gel.
- Loading blue dye:
  - Bromophenol blue 0,25 %
  - Cyanol xylene FF 0,25 %
  - Sucrose 40 %

  ↳ Keep at 4°C.

  ↳ Use diluted 6 times (2 µl of loading blue buffer for 10µl of PCR products).
- Molecular weight marker:
  - SmartLadder SF (Eurogentec): a ready-to-use molecular weight marker including 9 regularly spaced bands from 100 to 1000 bp.

4.4.2. Agarose gel preparation

1. Weight X g of agarose, add 100 x X ml of 1X TAE and heat until the mix is melted.
2. After cooling the solution, ethidium bromide is added (5 µl for 100 ml of agarose gel) and the solution is disposed in a specific mould equipped with combs (to make wells in the gel).
3. When gel is polymerised, combs are removed and the gel is placed in a horizontal electrophoresis system full of 1X TAE.
4. 10 µl of PCR products or 20 µl of RFLP products are mixed with 2 µl (for PCR products) or 4 µl (for RFLP products) of blue dye (6X) and disposed in the wells
5. One well is dedicated to the molecular weight marker (5 µl)
6. A voltage of 50 to 150 volts is applied during 30 min to 1 hour depending on the gel size and thickness
7. Gel is observed under UV.

6.3 Appendix C – Plankton samples

Table 6: Analysis of eukaryotic SSU rRNA genefragments in a metagenomic library constructed from planktonic (copepod) samples from Agapollen, containing high levels of infection of *Mytilus edulis* with *Marteilia pararefringens.* Analysis performed by Cefas, Weymouth.

Highest Taxon Rank			**No. of reads**	**Notes**
Alveolata	Apicomplexa		6	
	Ciliophora		421	
	Dinophyta		6	
Amoebozoa			6	
Chlorophyta			10	
Excavata			8	
Hacrobia			3	
Opisthokonta	Choanoflagellata		3	
	Fungi	Ascomycetes	7	
		Chytridiomycetes	10	
		Microsporidia	1	Microgemma
	Ichthosporea		1	86% match to Dermocstidium
Metazoa	Annelida		4	
	Arachnida		3	
	Branchiopoda		14	
	Malacostraca		54	
	Maxillopoda		1594	Principally *Acartia omorii*; other annotations: *Oithona brevicornis, Neocalanus cristatus, Centropages typicus, Eurycletodes laticauda, Calanus finmarchicus, Candacia truncate,* and *Maxillopod asp*.
	Cnidaria		7	
	Gastropoda		27	
	Unclassified		85	
Cercozoa			165	Including 6 paradinids (copepod parasites)
Stramenopiles	Bacillariophyta		41	
	Chrysophyceae		155	
	Labyrinthulea		4	
	MAST		4	
	Oomycetes		17	
	Blastocystis		2	