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The surveillance and control programme for *bonamiosis* and *marteiliosis* in European flat oysters, *Ostrea edulis*, and blue mussels, *Mytilus* sp. in Norway in 2017

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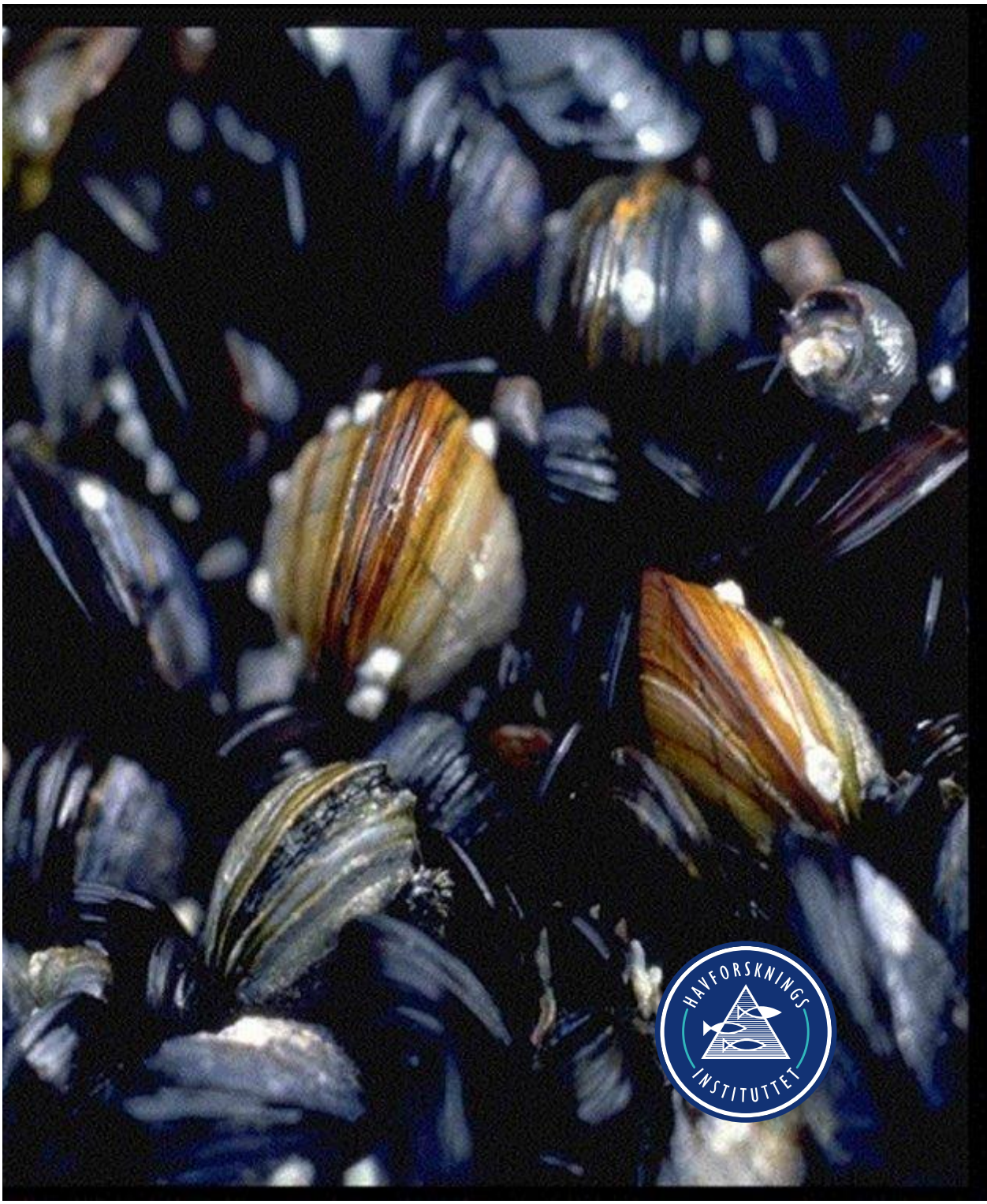

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Project Report

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The surveillance and control programme for *bonamiosis* and *marteiliosis* in European flat oysters, *Ostrea edulis*, and blue mussels, *Mytilus* sp. in Norway in 2017

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Summary (Norwegian):
Overvåkingsprogrammet for sykdommene bonamiose og marteiliose i flatøsters og blåskjell utføres av Havforskningsinstituttet på oppdrag fra Mattilsynet. Det ble hentet skjell fra fire ville bestander og ett østersanlegg, basert på utbredelsen av ville skjell og strukturen i østersnæringen. Prøver ble samlet inn i April/Mai og i Oktober, som er de periodene hvor prevalensen av parasittene *Bonamia* sp. og *Marteilia* sp. er høyest i smittede bestander. Det ble ikke observert unormal dødelighet verken vår eller høst. *Bonamia ostreae* / *B. exitiosa* ble ikke påvist. Det er kommet inn en rekke rapporter om at blåskjell «forsvinner» mange steder langs kysten. Årsakene til dette er ikke kjent. Parasitten *Marteilia* sp. ble imidlertid for første gang påvist I blåskjell, *Mytilus edulis,* på Bømlo i 2016. Denne påvisningen er fulgt opp med en utvidet prøvetaking i HI-prosjekt *Blåskjelldødelighet* (83737-04) i 2017. Det er gjort prøvetaking av blåskjell hver tredje måned og samlet inn fauna fra funnstedet og blåskjell fra områder i nærheten. Østers fra funnstedet ser ikke ut til å bli smittet av *Marteilia* sp. Genetiske studier av *Marteilia* spp. Fra England, Sverige og Norge (Bømlo) er inkludert i en studie som er gjort i EU-prosjektet VIVALDI. *Marteilia* sp. fra disse områdene er ulik *Marteilia refringens* som forårsaker sykdom hos flatøsters og er foreslått gitt navnet *Marteilia pararefringens*. Det ser således ut til at *Marteilia refringens* og *Marteilia pararefringens* sp. nov. er ulike arter med ulike vertsarter (hhv østers og blåskjell). Studiene videreføres i 2018, som en kombinasjon av overvåkingsprogrammet og forskningsprosjektet.

Summary (English):
The surveillance programme is carried out by the Institute of Marine Research according to a contract with the Norwegian Food Safety Authority. Samples were collected from four wild beds and one oyster farm, based on the present distribution of wild beds, and the structure of the oyster industry. Samples were collected in April/May and in October, in order to be able to detect *Bonamia* sp. and *Marteilia* sp. during the periods when the potential prevalence could be at the highest. No abnormal mortalities were observed in oyster populations during the surveillance. *Bonamia ostreae* / *B. exitiosa* were not detected during the surveillance programme in 2017. There have been several reports on mortality or “disappearance” of mussels along the Norwegian coast. The reason(s) for the mortalities have not been determined. However, the parasite *Marteilia* sp. was detected for the first time in mussels, *Mytilus edulis*. collected at Bømlo, western Norway in 2016. This has been followed up with an extended survey in the IMR project *Mussel mortalities* (83737-04), including a 3-monthly sampling of mussels at the site, associated fauna and mussels at nearby sites. A genetic study of *Marteilia* spp. from the UK, Sweden and the present site at Aga has been included in a study in the EU-project VIVALDI. The name *Marteilia pararefringens* has been proposed, and there is strong evidence that *Marteilia refringens* and *Marteilia pararefringens* sp. nov. are distinct parasites of bivalves and have different European distributions. Studies carried out so far indicate that *M. pararefringens* found in mussels does not infect flat oysters. The studies on *Marteilia pararefringens* sp. nov. are continued in 2018.

Emneord (norsk):
Østers, Blåskjell, *Marteilia pararefringens*, *Bonamia* sp., Overvåkning, Norge

Subject heading (English):
Oysters, Mussels, *Marteilia pararefringens*, *Bonamia* sp., Surveillance, Norway

Content

1 Introduction

Norwegian populations of European flat oysters, *Ostrea edulis*, have been considered free from notifiable diseases. In 2006, microcells resembling the oyster parasite *Bonamia* sp. were observed during histopathological examination of tissue specimens of flat oysters, *Ostrea edulis* from the Arendal area, southern Norway. In 2008, the EU reference laboratory received samples from the Norwegian Veterinary Institute, and reported one *Bonamia* sp. in a haemocyte from one oyster. By real-time PCR, positive results were obtained from two oysters in one triplicate sample. The parasite has however never been detected during examination carried out by the National Veterinary Institute or Institute of Marine Research. Since 2009, more than 2 600 oysters have been examined by histology and/or PCR, all with negative results. The situation has thus been stable since 2006 (see 2016 report and Mortensen *et al*. 2016).

The surveillance programme for bonamiosis and marteiliosis in European flat oysters, *Ostrea edulis*, and blue mussels, *Mytilus* sp. is carried out by the Institute of Marine Research according to a contract with the Norwegian Food Safety Authority. The programme was revised in 2015. The parasite *Marteilia* sp. was detected in mussels at one site in 2016, and we increased the effort in 2017 in order to study this case – including distribution and parasite life cycle.

This report gives a brief overview of the present situation, results from 2017 and suggestions for the 2018 sampling.

2 Material and methods

The surveillance was performed according to EU directive 2006/88 and Decision 2015/1554. The sampling strategy, including wild beds and bivalve farms in operation, was revised in January 2015, and used as a background for the targeted surveillance also in 2017.

Sampling periods were defined according to the periods when the highest prevalence of *Bonamia ostreae* and *Marteilia* sp. (sporulating stage) have been detected in the northernmost areas where they have been detected (Engelsma et al. 2010; A. Alfjorden pers.comm). The selected sampling sites are shown in Figure 1 and listed in Table 1.

At Hafrsfjord and Langestrand, oysters and mussels were collected by skin-diving or wading in April and October and transported to the Institute of Marine Research (IMR) in Bergen. At Sveio, oysters and mussels were collected by the shellfish farmer and sent to IMR Bergen by over-night mail (Table 1). From Ytre Hvaler, Østfold, mussels were collected by the local Nature Inspectorate (Statens naturoppsyn) and sent to IMR by over-night mail. At age, mussels and oysters were collected in the poll and brought directly to the laboratory in Bergen.

All oysters and mussels were processed at the IMR laboratory in Bergen, according to standard methodology, and under ISO 17025 QA. Briefly; Histology was performed using dorso-ventral cross sections, fixed in Davidson's fixative, embedded in paraffin, sectioned at 3µm, stained with Hematoxylin Eosin Saffron (HES), mounted with a cover slip and observed at 100 to 1000 x magnification.

Samples where microcells were observed by microscopy will be forwarded to real-time PCR as described by Marty *et al*. 2006 and Corbeil et al 2006.

After the observation of *Marteilia* sp. by histology, DNA was extracted from ethanol fixed digestive gland tissue from mussels from Aga. *Marteilia refringens* detection and typing was done with by Real-time Polymerase Chain Reaction, and as described by Le Roux et al (2001).

Mussel tissue samples fixed in ethanol were sent to CEFAS, Weymouth, UK, and included in a study on the speciation of mussels and *Marteilia* spp. from Northern Europe.

Thirty mussels for histological examination were sampled in April, July, October and January (2018) respectively, thus representing a full year cycle of *Marteilia* sp.

Fauna samples and additional samples from mussels and oysters were collected in July (Table 2). Additional fauna samples were collected in October. Samples from July were processed and analyzed by PCR as described above, at IMR or CEFAS.

Figure 1. Yellow circles indicate the sampling sites for flat oysters (*Ostrea edulis*) and mussels (*Mytilus* sp.). The blue circle indicates the sampling site at Ytre Hvaler, where mussels were collected in October.

Table 1. Sampling and surveillance of flat oysters (*Ostrea edulis*) and mussels (*Mytilus* sp.) in 2017.

Sampling site	**Oysters**		**Mussels**	
	Spring	Autumn		
Ytre Hvaler, Østfold			30 (10)	30 for PCR, 10 for histology
Langestrand, Aust-Agder	150 30	30	30	
Hafrsfjord, Rogaland	30	30	30	
Sveio, Hordaland	30	30	30	
Aga, Bømlo, Hordaland	30	30	30	Extra samples summer and winter, see M&M section

3 Results

Bonamia spp. was not observed in any sample during 2017.

Langestrand, Aust-Agder.
The site was inspected by skin diving in May 2017. Dense oyster beds were observed down to approximately four-meter depth, with several cohorts present. There was no sign of abnormal mortality. Few adult Pacific oysters (*Crassostrea gigas*) were observed between the flat oysters. During sampling, Pacific oyster spat were observed on and in-between flat oyster shells and on pebbles in the inter-tidal zone.

Oysters: During examination of the flat oysters, gross morphology of shells and soft parts appeared normal. *Bonamia ostreae* / *B. exitiosa* or microcells resembling *Bonamia* spp. were not detected. *Mikrocyto*s-like cells were observed in two oysters. These were forwarded to PCR analysis. Intracellular Rickettsia-like organisms (RLO's) were observed in the digestive tissues of seven oysters.

Also during sampling in October, there was no sign of abnormal mortality. Microcells were not observed during the histological examination. The oysters appeared in good health.

Mussels appeared normal, however most specimens had green pustules, presumably representing infections with the parasitic algae *Coccomyxa parasitica* (see Mortensen *et al*. 2005). *Marteilia* sp. was not observed.

Hafrsfjord, Rogaland
Samples were collected at Sørnes in May and October (Table 1). Dense, patchy oyster beds were observed down to approximately three-meter depth, with several cohorts present. There was no sign of abnormal mortality. A few adult Pacific oysters (*Crassostrea gigas*) were observed between the flat oysters on shallow water. *Bonamia* sp. or *Marteilia* sp. were not observed in mussels or oysters. During examination of the flat oysters, perforations due to *Polydora* sp. infestations were observed in shells from all oysters. Gross morphology of soft parts appeared normal. RLO's were observed in three oysters. Haemic neoplasia was observed in three oysters.

Sveio, Hordaland
Oysters: Two *Mikrocytos*-like cells were observed in one oyster. These will be forwarded to PCR-examination. Haemic neoplasia was observed in one oyster and one mussel.

Ytre Hvaler, Østfold
Mussels received from Ytre Hvaler were too small for a proper examination. The ten largest ones were processed for histological examination. These appeared in good health. All 30 will be analyzed by Marteilia- PCR.

Aga, Bømlo, Hordaland: Studies on the *Marteilia* infection

Oysters: The condition index of the oysters was low. Haemic neoplasia was observed in six oysters, and Rickettsia-like colonies (RLO's) were observed in the digestive epithelia of six oysters. *Marteilia* sp. was not observed and the *Marteilia*-PCR (spring) was negative (see Table 2). A few *Mikrocytos*-like cells were observed in one oyster. This will be forwarded to PCR-examination.

Mussels: The mussels at Aga were *Mytilus edulis* (D. Bass, pers. comm). *Marteilia* sp. detected in the mussels was *Marteilia pararefringens* sp. nov (Kerr *et al*. in press).

Marteilia pararefringens sp. nov was detected in mussels in the Aga poll and Håpollen (connecting the Aga poll with the outside fjord). Prevalence in the mussels collected at Aga varied between 30 and 70 %. Young parasite stages were observed all year. Sporulating stages were observed only in the autumn sample (October).

Mussels from Rogøysund (receiving oyster spat from Aga), Kulleseid (wild population, Bømlo) and Kvalvågnes, Lindås (wild population collected on an abandoned oyster farm that previously received oyster spat from Aga) were negative. From the first fauna sampling, plankton samples and samples from the digestive gland of shrimp (*Palaemon* sp.) were positive (Table 2).

Table 2. Mussels, flat oysters and fauna sampled in July 2017 were analyzed by a *Marteilia*-specific PCR. additionally, samples analyzed at CEFAS were analyzed in Haplosporidia and general Paramyxa PCR-assays. *Marteilia*-positive samples are marked in red.

Site	Species	Number	Haplosporidia	Paramyxid	Marteilia ITS
Aga infected site	Mussels	1-30			8/30 + 4/10
	Oysters	1-30			0/30
	Shrimp, *Palaemon* sp.	1-30	0/30	0/30	5/30
	Periwinkles, *Littorina littorea*	1-30	0/30	0/30	0/30
	Plankton / copepodes	6 pools	0/6	6/6	5/6
Håpollen, nearby	Mussels	1-30	0/30	15/30	5/30
Kulleseid, Bømlo distance	Mussels	1-30	0/30	0/30	0/30
Rogøysund; oyster farm receiving spat from Aga, distance	Mussels	1-30	0/30	0/30	0/30
	Oysters				0/30
Lindås; at oyster farm receiving spat from Aga years ago, distance	Mussels				0/30

4 Discussion and conclusions

Examination of flat oysters

The wild flat oyster populations examined appears healthy, with a normal reproductive cycle pattern. Haemic neoplasia and the presence of intracellular Rickettsia-like colonies were occasionally observed, but at low prevalence and intensity. This is a common observation, and not considered a problem, although the neoplasia may cause problems and potentially induce winter mortalities of flat oysters in severe cases (Mortensen *et al*. 2013).

The oysters from Aga appear in relatively poor condition, with low condition index. This is probably due to food limitation. At Langestrand, several cohorts have been present throughout the study period. All samples since 2008 have been *Bonamia* negative (Mortensen *et al*. 2016). The situation has thus been stable since 2006. A 12 years long sub-clinical *Bonamia* infection seems unlikely, taking into account that this oyster bed experiences extremely variable conditions through the seasons. We consider the bivalves examined in 2017 as negative with regard to *Bonamia ostreae* / *B. exitiosa*. Autumn sampling was reduced to 30 oysters, in accordance with EURL recommendations (I. Arzul, pers. comm).

Marteilia spp. has not been detected in oysters by histological examination. The oysters collected in the poll, close to the *Marteilia*-infected mussels, were histology negative but PCR positive in October. Sequencing showed that also the oysters had *M. refringens* type M. / *Marteilia pararefringens* sp. nov. Oysters were however PCR-negative in April. The positive PCR signals in October correspond to the time of *M. pararefringens* sporulation in the mussels, and may be due to filtration og spores released from the infected mussels. Oysters and mussels collected at Rogøysund – a farm receiving oyster spat from Aga, were negative, indicating that *M. pararefringens* has not been moved to oyster farms with oyster spat from the poll.

Examination of mussels

The detection of *Marteilia* sp. (now proposed *Marteilia pararefringens* sp. nov.) still appears surprising. Samples collected by the Norwegian Veterinary Institute in 2014 were negative. These samples from 2014 should be re-examined and the sampling date used in an analysis of the occurrence of the parasite in the mussel population.

Other studied mussel populations appear free from *Marteilia* sp. However, the number of populations is still too low to give an overview of the situation. More sites should be included in the surveillance, including selected sites in the mussel producing areas in Trøndelag.

Distribution of *Marteilia pararefringens* sp. nov.

M. pararefringens has so far only been detected in Aga. Mussels collected in Håpollen, approximately one km from the Aga poll were PCR positive. This should be followed up with sampling in a distance gradient from the infected site. A report of a *Marteilia* detection near Stavanger in 2010 (Arab *et al*. 2011) should also be followed up in 2018.

The Aga poll has been used to produce flat oyster, *Ostrea edulis*, spat since 1884, and the lagoon and a nearby site was the center for an integrated production of flat oysters in the 1990's, organizing around 40 oyster farmers in a network. It is important to map all historical movements of oyster spat in and out of the site, in order to design an extended sampling scheme for an epidemiological survey. Additionally, wild mussels have to be examined in an

increasing distance from the infected site. In 2017, this work included one farm that is annually receiving spat from Aga (Rogøysund) and one farm that received oyster spat from Aga many years ago (Lindås). Further studies are needed in order to examine if *Marteilia* is limited to Aga or more widely distributed. Further studies (in 2018) will include other oyster polls as well as wild mussel populations near the infected site.

Preliminary data supporting a study of the life cycle of *Marteilia pararefringens* sp. nov.

To understand the spreading potential of *M. pararefringens*, we need to understand the life cycle of the parasite. Histopathological examination of mussel tissues revealed young stages in stomach and tubule epithelia throughout the year. Maturation into secondary stage was occasionally observed in the stomach epithelium, but mainly in digestive diverticulae. Parasites in a mature, sporulating stage was observed in October. These data represent a good starting point for the study on the life cycle of *M. pararefringens* in Norway.

It is also crucial to investigate which (if any) organism(s) that may act as intermediate host(s). We therefore started a study at the infected site, screening fauna for *Marteilia*-DNA. Samples from shrimp (*Palaemon* sp.) and copepods collected in July were PCR-positive. For *Marteilia refringens* in oysters, copepods (*Paracartia grani*) are proposed as intermediate hosts (Audemard et al. 2002; Boyer *et al*. 2013). We plan a more detailed examination of the positive copepods as well as a new sampling to elucidate the role of the copepods and the shrimp. This work will be planned and designed when the results from fauna sampling in October 2017 are ready.

To study the time of infection and the potential need for an intermediate host to complete the life cycle, experimental transmission trials will be carried out in the field, as well as in the IMR laboratory facilities.

Studies on *Marteilia* sp. from Northern Europe reveal new insight to this genus. There is strong evidence that *Marteilia refringens* and *Marteilia pararefringens* sp. nov. are distinct parasites of bivalves and have different European distributions (Kerr *et al*. in press). The life cycle of *M. pararefringens* is however unknown. The poll represents an excellent study site, and studies trying to reveal the parasite life cycle will be continued in 2018. Studies will also be carried out in other oyster polls, in order to elucidate if the Aga case is unique, or if *M. pararefringens* is present also in other, similar environments.

It is important to be sure that oysters are not susceptible to *M. pararefringens* or may act as vectors. We will thus combine surveillance and research activity in order to obtain as much data as possible, also from oysters at the infected site, and from more sites that have been in contact with the former network of oyster producers.

We will discuss a new revision of the surveillance programme, including the main mussel producers in Trøndelag and propose a model for a regional health surveillance in this area.

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