



THE SURVEILLANCE AND CONTROL PROGRAMME FOR BONAMIOSIS AND MARTEILIOSIS IN EUROPEAN FLAT OYSTERS, *OSTREA EDULIS*, AND BLUE MUSSELS, *MYTILUS SP.* IN NORWAY IN 2018

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Tittel (norsk og engelsk):

The surveillance and control programme for bonamiosis and marteiliosis in European flat oysters, *Ostrea edulis*, and blue mussels, *Mytilus* sp. in Norway in 2018

Overvåkings- og kontrollprogram for bonamiose og marteiliose i flatøsters, **Ostrea edulis**, og blåskjell, **Mytilus** sp. i Norge i 2018 Rapport NOK østers 2018

Rapportserie:

Rapport fra

Havforskningen

ISSN:1893-4536

År - Nr.:

2019-17

Dato:

30.04.2019

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Distribusjon:

Åpen

Prosjektnr:

14538 & 83737-04

Oppdragsgiver(e):

Mattilsynet

Oppdragsgivers referanse:

Jonathan Vaz

Program:

Akvakultur

Kystøkosystemer

Faggruppe(r):

Sykdom og smittespredning

Antall sider:

16

Sammendrag (norsk):

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, two mussel farms and one oyster farm. 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. The results may be used as a background for an application for disease free status for Norwegian flat oysters.

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* at Bømlo, western Norway, collected during the surveillance programme in 2016. This has been followed up with an extended survey in the IMR research project *Mussel mortalities* (83737-04). We have performed transmission experiments that identify the time period of infection and the progress of the infection in mussels. Analyses of samples collected in July 2018 indicate that the parasite may be present in an abandoned oyster lagoon at Espevik, Tysnes.

The results from the research project indicate that the *Marteilia* sp detected is limited to mussels. Flat oysters at the same site do not become infected. This is relevant to the listing of susceptible hosts for *Marteilia* spp. 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. After the detection of *M. pararefringens* in mussels, it is important to obtain more data from mussels along the Norwegian coast. Mussels from Trøndelag in 2018 represented the first samples from mussels north of Bergen. In order to obtain a better set of data, we propose an extended surveillance that could be obtained through a revised surveillance programme combined with a new model for health control in mollusk farms.

Sammendrag (engelsk):

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, to blåskjellanlegg og ett østersanlegg. Høsten 2018 ble det for første gang inkludert prøver fra blåskjell fra Trøndelag. Prøver ble samlet inn i April/Mai og i Oktober, når prevalensen av parasittene *Bonamia* spp. og *Marteilia* spp. er høyest i smittede bestander. Det ble ikke observert unormal dødelighet verken vår eller høst. *Bonamia ostreae* / *B. exitiosa* ble ikke påvist. Resultatene kan danne bakgrunn for en søknad om etablering av fristatus for *Bonamia* spp. i norsk flatøsters.

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 *Blåskjell dødelighet* (83737-04) i 2017 og 2018. Det er gjort smittestudier som gir informasjon om smittetidspunkt og -forløp. Prøver samlet inn i Juli 2018 tyder på at parasitten også kan finnes i den nedlagte østerspollen i Espevik, Tysnes.

Resultatene viser at infeksjonen med *Marteilia* sp. er begrenset til blåskjell. Østers fra den samme lokaliteten blir ikke smittet. 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). På bakgrunn av funnet av *M. pararefringens* er det viktig å skaffe mer informasjon om helsestatus hos blåskjell langs hele kysten. Dette kan gjøres gjennom en utvidet helseovervåking, eventuelt gjennom en ny modell hvor overvåking og helsekontroll kobles, for å generere mer data.

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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 Norwegian Veterinary Institute or Institute of Marine Research. Since 2009, more than 2 700 oysters have been examined by histology and/or PCR, all with negative results. The situation has thus been stable since 2006 (see 2017 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 and 2018 in order to study this case – including distribution and parasite life cycle.

This report gives a brief overview of the present situation, results from 2018 and suggestions for the 2019 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 2018.

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 and sent to IMR by over-night mail. At Aga, mussels and oysters were collected in the poll and brought directly to the laboratory in Bergen. Additional samples were taken from fauna. Samples from plankton and mussels were also collected in two other oyster lagoons; Innerøypollen (Halhjem) and Espevikpollen (Tysnes). In 2018, mussels from the two dispatch centers processing mussels in Trøndelag were included for the first time. Trøndelag is the main production area for mussels in Norway.

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 for PCR were fixed in ethanol. DNA was extracted from ethanol fixed digestive gland tissue from mussels from Aga. *Marteilia refringens* detection and typing was done with by Polymerase Chain Reaction, and as described by Le Roux et al (2001). Samples where (*Bonamia*-like) microcells were observed by microscopy were forwarded to real-time PCR as described by Corbeil et al 2006.

Healthy mussels were deployed at the infected site in May and October. Thirty mussels were sampled every six weeks until April 2019 and analyzed by histology and PCR.

Fauna samples and additional samples from mussels and oysters were collected in May, July and October. Samples were processed and analyzed by PCR as described above, at IMR or CEFAS, UK.



Figure 1. Sampling of flat oysters, *Ostrea edulis*, and blue mussels, *Mytilus edulis* in the surveillance programme for *Bonamia* sp. and *Marteilia* sp. Yellow circles indicate the sampling sites for flat oysters. Blue circles indicate the sampling sites for mussels.

Table 1. Sampling and surveillance of flat oysters (*Ostrea edulis*) and mussels (*Mytilus* sp.) in 2018.

| Sampling site | Oysters | | Mussels | | |
|--|----------------|--------|---------|--------|---|
| | Spring | Autumn | Spring | Autumn | |
| Ytre Hvaler, Østfold | | | 30 | | |
| Langestrand, Agder | 150 (20) 30 | | 30 | | NB: delayed reading of slides. Histo & PCR (30) spring |
| Hafrsfjord, Rogaland | 30 | 30 | 30 | | Histo & PCR (30) spring |
| Sveio, Hordaland | 30 | | | | |
| Aga Bømlo, Hordaland Rogøysund, Bømlo | 30 | 30 | 10 | | Additional study of <i>Marteilia pararefringens</i> in mussels at Aga, see text |
| Innerøyen, Hordaland | | 30 | | | July, PCR |
| Espevik, Tysnes, Hordal. | | 30 | | | July, PCR |
| Åfjord, Trøndelag | | | 30 | | |
| Rissa, Trøndelag | | | 30 | | |

3 - Results

Bonamia spp. was not observed in any sample during 2018.

Langestrand, Aust-Agder.

The site was inspected by skin diving in May 2018. 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: The reading of slides from oysters is delayed (see Table 1). During examination, gross morphology of shells and soft parts appeared normal. In the first 20 slides *Bonamia ostreae* / *B. exitiosa* or microcells resembling *Bonamia* spp. were not detected.

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.

Hafersfjord, Rogaland

Samples were collected in May and October (Table 1). Sampling was performed by Knut Magnus Persson in conjunction collection of flat oysters for a re-stocking programme off the coast of The Netherlands. No sign of abnormal mortality was reported. 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. *Bonamia* PCR of oysters were negative. During examination of the flat oysters, perforations due to *Polydora* sp. infestations were observed in shells from most of the oysters. Gross morphology of soft parts appeared normal. Rickettsia-like organisms (RLO's) were observed in three oysters. Haemic neoplasia was observed in one oyster. The condition of the mussels was variable. Focal inflammations that may represent infections with *C. parasitica* was observed in five mussels.

Sveio, Hordaland

Oysters sampled in spring appeared healthy and in good condition. The dispatch centre at Sveio (Sunnhordland havbruk) has been closed, and the site was excluded from the surveillance programme after the spring sampling.

Ytre Hvaler, Østfold

Mussels from Ytre Hvaler (Papperhavn) were in variable condition. Microcells were observed in connective tissues, in some cases associated with inflammations. Green spots were observed in mantle tissues of all mussels, and the infestations were considered infections with *C. parasitica*. Most mussels were moderately infected with trematode cysts. Un-known microcells were observed inside cells in the

stomach epithelium. Histological slides and tissue fixed in ethanol was forwarded to the Community Reference Laboratory for further investigation.

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

Oysters: The condition index of the oysters was variable. Haemic neoplasia was observed in one oyster, and Rickettsia-like colonies (RLO's) were observed in the digestive epithelia of three oyster. *Marteilia* sp. was not observed and the *Marteilia*-PCR (spring and autumn) were negative.

Mussels: *Marteilia pararefringens* sp. nov was detected in mussels in the Aga poll in all samplings. A focused study on the infection cycle in mussels was carried out with sampling every 6 weeks from May 2018 to April 2019. The results from this study will be presented separately, as a master thesis, later as a publication. Mussels from Rogøysund (receiving oyster spat from Aga and also used in this study as negative control), were negative.

Analyses of mussels from two other lagoons; Innerøyen and Espevik

PCR performed on mussels from Innerøypollen was negative. However, when PCR was conducted on mussels from Espevikpollen (Tysnes), two mussels were *Marteilia*-positive.

Åfjord, Trøndelag

Mussels from Åfjord appeared in good condition. No abnormal finding was recorded.

Rissa, Trøndelag

Mussels from Rissastrømmen appeared in good but variable condition. Green colonies (*C.parasitica*) was observed in the mantle of two mussels. No other abnormal finding was recorded.

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 13 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 2018 as negative for *Bonamia ostreae* / *B. exitiosa*.

Marteilia spp. has not been detected in oysters by histological examination. The oysters collected in the lagoon at Aga, close to the *Marteilia*-infected mussels, were PCR negative. 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 and the distribution of *Marteilia pararefringens* sp. nov.

M. pararefringens has so far only been detected in Aga. Mussels collected in Håpollen in 2017, approximately one km from Aga were PCR positive. 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, where two dispatch centers receive mussels from several producers in a large geographic area. This illustrates the need for combining the surveillance with the recently started health control of shellfish farms. A report of a *Marteilia* detection near Stavanger in 2010 (Arab *et al.* 2011) should be followed up in 2019.

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. Further studies are needed in order to examine if *Marteilia* is limited to Aga or more widely distributed. The study carried out in 2018 included two other oyster lagoons. Two PCR positive samples from the abandoned oyster lagoon at Espevik, Tysnes, indicate that *Marteilia* sp. is present at this site. A new sampling will be performed in July 2019, in order to verify the result by histology.

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

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.* 2018). The life cycle of *M. pararefringens* is however unknown. To understand the spreading potential of *M. pararefringens*, we need to understand the life cycle of the parasite. Histopathological examination of mussel tissues in 2017 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. However, the mussels were 2-3 years old, and may have been infected several times. Two deployments of healthy mussels on the infected site in 2018 revealed the time window of infection, the progress of the infection in mussels, as well as the time of sporulation. These data represent a good starting point for the study on the life cycle of *M. pararefringens* in Norway.

The life strategy of *M. pararefringens* from the time of sporulation (autumn) until re-infection of mussels (summer) is still un-known. PCR-analysis of fauna samples gave positive results in samples from plankton sampled during summer. The studies will be continued in 2019, focusing on plankton, other invertebrate fauna, water and sediment in the period May – July.

Our data indicate that flat oysters are not susceptible to *M. pararefringens*. It is important to be sure that oysters may not act as vectors. We will thus continue to 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.

Conclusions

Bonamia spp. has not been detected during the surveillance programme. We are still not able to detect *Bonamia* spp. at Langestrand and recommend that the sample size is reduced. The results may be used as a background for an application for disease free status regarding *Bonamia ostreae* and *B. exitiosa* in this zone, and a revision of the categorization of zones.

Marteilia pararefringens has been detected at Aga, however additional samples from July 2018 indicate the presence of this parasite also in mussels at Espevik, another former oyster lagoon. The studies of *M. pararefringens* will be continued in order to describe the life cycle as well as the distribution of the parasite. The data from 2018 indicate that flat oysters are not susceptible to *M. pararefringens*.

The samples from Trøndelag collected in 2018 represent the first study of the health status of mussels north of Bergen. The lack of information on mussels from most of the Norwegian coast illustrate the need for an extended surveillance or a new model for health monitoring of mussels and oysters which combines surveillance and health control.

Acknowledgements

Thanks to Ingrid U. Fiksdal, Dawit B. Ghebretnsae and Håkon Berg-Rolness for technical assistance, to Knut Magnus Persson for help with sampling in the field and to David Bass for the collaboration on the *M. pararefringens* life cycle.

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