THE SURVEILLANCE AND CONTROL PROGRAMME FOR BONAMIOSIS AND MARTEILIOSIS IN EUROPEAN FLAT OYSTERS, *OSTREA EDULIS*, AND BLUE MUSSELS, *MYTILUS* SP. IN NORWAY IN 2020

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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 2020

Overvåkings- og kontrollprogram for bonamiose og marteiliose i flatøsters, *Ostrea edulis*, og blåskjell, *Mytilus* sp. i Norge i 2020

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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 one oyster farm, four wild oyster populations, one cultivated mussel population and two wild mussel beds. 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 is at the highest. No abnormal mortalities were observed during the surveillance. *Bonamia ostreae* / *B. exitiosa* were not detected. 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 mussela, *Mytilus edulis*, collected at Bømlo, western Norway in 2016 and Tysnes in 2019. In 2020, *Marteilia* was detected in wild populations in Sunnfjord and in an open water mussel farm nearby. All detections were *M. pararefringens* . The work will be continued in 2021, linked to research on the distribution of *M. pararefringens* in wild mussels. We propose a revision of the surveillance programme combined with the establishment of a new model for health control in mollusk farms, application for disease free status for Norwegian flat oysters and a categorization of zones.

Summary (Norwegian):

Overvåkingsprogrammet for sykdommene bonamiose og marteiliose i flatøsters og blåskjell utføres av Havforskningsinstituttet på oppdrag fra Mattilsynet. Det ble hentet skjell fra ett dyrkingsanlegg for østers og fire ville østersbestander, en kultivert blåskjellbestand og to ville blåskjellbestander. Prøvene ble samlet inn i de periodene prevalensen av parasittene *Bonamia* spp. og Marteilia spp. er vist å være høyest i smittede bestander. Det er 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 ville blåskjell, Mytilus edulis, på Bømlo i 2016, og på Tysnes i 2019. I 2020 ble det gjort funn av denne parasitten i ville bestander i Sunnfjord og i dyrkede blåskjell i nærheten av de ville bestandene. Alle funn av *Marteilia* i blåskjell i Norge er så langt *Marteilia pararefringens*. Resultatene fra overvåkingen vil bli fulgt opp med en utvidet prøvetaking av ville blåskjellbestander i 2021 – 2022 i regi av forskningsprosjektene. Det foreslås en revisjon av overvåkingsprogrammet i sammenheng med etablering av en modell for helseovervåking av skjellanlegg, søknad om etablering av fristatus for *Bonamia* spp. i norsk flatøsters og en gjennomgang av kategoriseringen av soner.

Content

1 Introduction

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

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 over-all aim is to gain knowledge on the health situation of farmed Norwegian oysters and mussels. There is however a connection between farmed and wild populations, due to the geographically widespread and extensive nature of the bivalve industry. The work therefore requires a tight link between the Surveillance programme and the on-going research projects, as well as the building of a time-series of data on wild stocks. After the detection of *Marteilia* sp. in 2016, we have increased the effort to study this case – including distribution and parasite life cycle. This report gives a brief overview of the present situation, results from 2020 and the prospects for the work in 2021.

Norwegian flat oyster, Ostrea edulis, are used in re-stocking projects

There is a growing interest in the re-establishment of wild oyster beds in Northern Europe, and initiatives united in the NORA project (https://noraeurope.eu/) now work to develop a protocol on how to select, treat and seed oysters on some of the historical oyster beds. We are collaborating with this project, with contributions to their biosecurity manual and linking the selection of potential oyster source population to the on-growing health monitoring and research.

Both the oyster farming industry and re-stocking projects focus on where to find naïve flat oyster populations that are free from *Bonamia* spp, as well as other pathogens that may affect the populations. In the present situation – and after the re-occurrence of *Bonamia ostreae* in Limfjorden, Denmark, in 2014 – safe sources of oysters can probably only be found in Sweden and Norway. Norwegian populations of European flat oysters have been monitored since 1989 and are considered free from notifiable diseases (Mortensen et al. 2016; 2020). Oysters from Hafrsfjord in Rogaland are being used in re-stocking trials in the North Sea. Oysters from this area are included in the surveillance program.

Marteilia pararefringens sp. nov. in blue mussels, *Mytilus edulis* in Norway

The blue mussel, *Mytilus* sp, populations are changing, and there are numerous reports on an un-explained “disappearance” or mortalities from many Nordic areas. It is not known to which extent diseases play a role in these changes. The wild mussel beds are not monitored on a regular basis, and there is a limited control of mussel farms, using traditional longline cultivation based on the collection of wild seed.

Marteilia pararefringens has been found in blue mussels, *Mytilus edulis*, in two traditional heliothermic marine oyster lagoons in western Norway. These lagoons have much higher water temperatures than the ambient fjord water during the summer season, and have been used for breeding oysters since the 1880’s. The semi-closed system of the oyster ponds represents a unique opportunity to study the life cycle and parasite progression of *M. pararefringe*ns in *M. edulis*. We use the pond at Aga, on the island of Bømlo, Western Norway, as a study site to elucidate the infection and life cycle of *M. pararefringens* in mussels.

M. pararefringens have never been detected during our health surveillance of mussels from open water farms or wild mussel beds along the coast. There is however one report of *Marteilia* sp. was observed in blue mussels, *Mytilus edulis* used for environmental monitoring around 2010 (Aarab et al. 2011), but this finding was not notified. In 2020, the Norwegian Veterinary Institute reported *Marteilia*-infected mussels from a health control in an open-water farm. Our sampling in 2020 was therefore extended to include mussels from wild mussel populations in the vicinity of this farm.

2 Material and methods

The surveillance was performed according to EU directive 2006/88 and Decision 2015/1554.

Sampling periods were defined according to the periods when the prevalence of *Bonamia ostrea*e and *Marteilia* sp. (sporulating stage) are highest 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.

Usually, the surveillance includes an on-site survey, as the state of the population (density, reproduction, signs of mortality) are considered important meta-data. At Hafrsfjord, Haugevågen, Innerøyen, Flekke and Ostrevig oysters and mussels were collected by skin-diving or wading and transported to the Institute of Marine Research (IMR) in Bergen. At Strømmen the mussels were collected by personnel from the Food Safety Authority. At Vågstranda, mussels and oysters were collected by the local landowner and sent to IMR Bergen by over-night mail (Table 1). At Langesand, the site was inspected by skin diving. Samples were not collected, as this site is no longer included in the surveillance programme.

All oysters and mussels were processed at the IMR laboratory in Bergen, according to standard methodology, and under ISO 17025 QA. The samples with 100 specimens were split in two. Half were analyzed using histology, the other half with Polymerase Chain Reaction analysis (PCR). Samples from Flekke, Haugland (Table 1) were analyzed in parallel. 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. Marteilia detection and typing was done with by PCR as described by Le Roux et al (2001). Positive samples were sequenced.

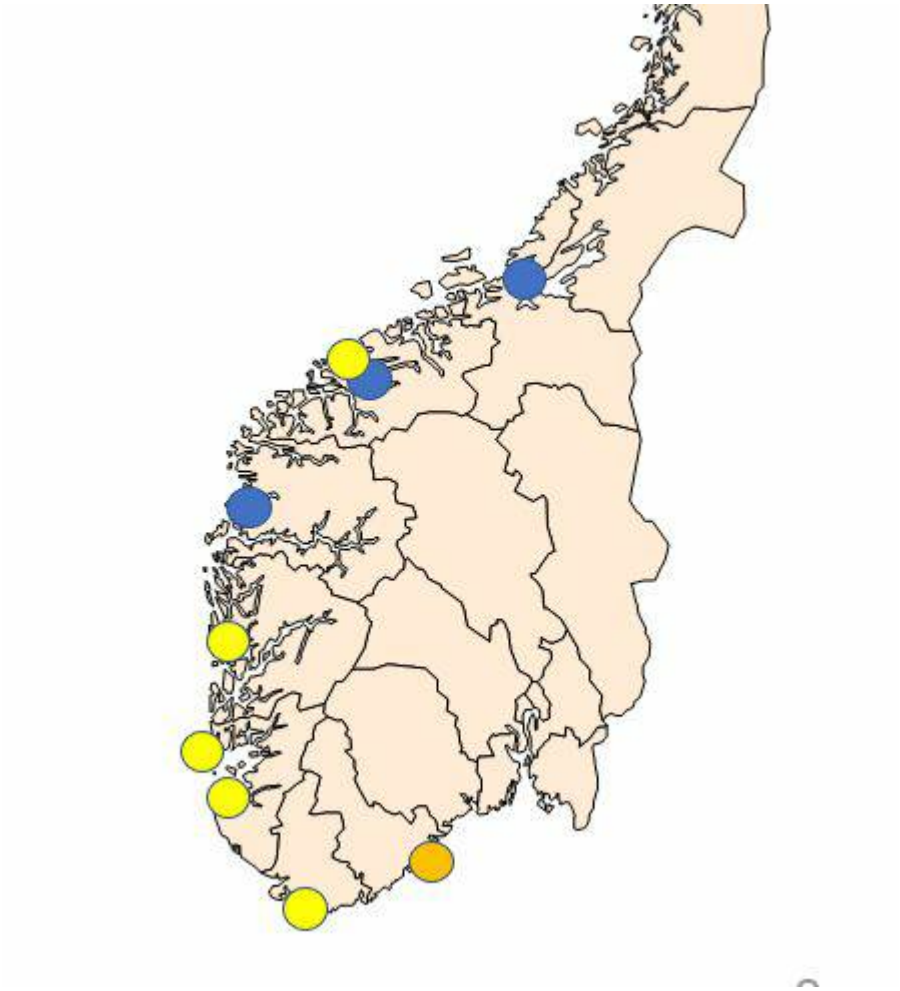

Figure 1. Sampling sites of flat oysters, *Ostrea edulis* (yellow) and blue mussels, *Mytilus* sp. (blue) in the surveillance programme for *Bonamia* sp. and *Marteilia* sp. See also Table 1. The flat oyster population at Langesand (orange) was inspected but not sampled in 2020.

Table 1. Sampling and surveillance of flat oysters (Ostrea edulis) and mussels (Mytilus sp.) in 2020.

Sampling site	**Oysters**		**Mussels**		
	Spring-summer	**Autumn**	**Spring**	**Autumn**	
Hafrsfjord, Rogaland	100				52 obs by microscopy, 48 by PCR
Haugevågen, Rog.	99				51 by microscopy, 48 by PCR
Innerøy, Vestland	30				PCR only

Ostrevig, Rogal.		30			Microscopy only
Vågstranda		18		22	Microscopy only
Flekke, river				50	30 obs by histology
Flekke, Haugland				50	30 obs by histology, all by PCR
Strømmen, Rissa, Trøndelag				100	52 obs by microscopy, 48 by PCR

3 Results

Bonamia spp. was not observed in any sample in 2020. *Marteilia* sp. was not observed in oysters but *M. pararefringens* were present in mussels from two sites in Flekke. Results from the sites listed in Table 1 are briefly described below.

Examination of flat oysters

Langesand (58.539225, 8.937646)

Langesand has a large population of flat oysters. The site was previously included in the surveillance programme and subjected to a targeted *Bonamia* survey (see previous reports).

The site has an apparent stable sub-population of flat oysters growing from 3-6 m depth mostly protected from harvesting and climatic events. From 3m to surface the population has variable recruitment success and survival due to predation, harvesting and ice conditions. Both sub-populations contain specimens of several year-classes. There was no sign of elevated mortality. Oysters were not sampled for further examination in 2020.

Hafrsfjord (58.926864, 5.6436861)

Hafrsfjord is located west of the city of Stavanger in Rogaland county, Western Norway. The fjord has a narrow inlet and appears as a semi-closed marine fjord-system. Hafrsfjord has a large population of flat oysters. These oysters have been harvested and used in re-stocking projects in the North Sea. Six sites in Hafrsfjord were visited in June 2020. At five sites, low to moderate densities and several year-classes of flat oysters were observed. Oysters observed by microscopy appeared normal, had a good condition and variable gonad status. Some shells were infested by *Polydora* sp. Haemic neoplasia was observed in two oysters.

Haugevågen, Rogaland (59.3665555, 5.2489903)

Haugevågen is a sheltered lagoon on the north-western side of the island of Karmøy, in Rogaland. The lagoon and inlet have a dense population of flat oysters, down to 4 – 5 m depth. Oyster farmers have applied for a permission to use this site to collect flat oyster spat, and a visit was performed to gain information and data on the oyster population there. The population is dominated by a few strong year-classes. There are patches of mussels in some areas along the shoreline. A few Pacific oysters were found and removed. The site was visited in June. Oysters had a good condition, and most were sexually mature. Some were in a state of spawning. Rickettsia-like organisms (RLOs) were observed in the digestive gland of six oysters. Haemic neoplasia was observed in three oysters.

Innerøy (60.1508985, 5.4122928)

Innerøyen is presently the only site in Norway with a traditional production of flat oyster spat.

Oysters were collected from suspended ropes in the mid part of the lagoon in July. The condition index of the oysters was low but variable. All samples were *Bonamia* negative by PCR analysis.

Ostrevig (58.3271717, 6.2350926)

Ostrevig is a traditional oyster lagoon that was abandoned in the 1990's. The lagoon has no direct contact with the sea. Seawater splashes over the rocks on high tide and windy weather and fills the lagoon. There is an isolated population of flat oysters in the lagoon. Oysters were sampled for genetical studies and histology. The oysters had a low condition index but normal digestive epithelia, and passive / empty gonadal tissues. Early-stage haemic neoplasia was observed in two oysters. In one oyster, we observed heavy secretion of eosinophilic granules in the digestive tissues, indicating a lysosomal destabilization, probably as an effect of an external stressor.

Vågstranda (62.6040173, 7.3325886)

Vågstrandapollen (Vågen) is an approximately 1,8-kilometer-long estuary connected to the Romsdal fjord by a shallow sill with a tidal current. The estuary is sheltered and has been used as an oyster lagoon from the 1930's until the 1990's. The site is abandoned, but there is a remaining population of flat oysters in the estuary. Oysters were sampled during winter. Sections from 22 oysters were observed by microscopy. All oysters appeared healthy. Gonads were empty. Some damage was observed on the gills, presumably due to low temperatures before shipment. RLOs were observed in one oyster.

Examination of Mussels

Flekke (61.3134761, 5.3467415)

After the detection of *Marteilia* sp. in mussels in an open water longline farm, we surveyed two local mussel populations in the Flekke fjord:

1. There is a mussel population in the Flekke river estuary, in the river mouth and along hard bottom areas. In the river mouth we found only large mussels in a low density on the rocks, and a mussel bed on shallow water along the west side, with a high number of empty/dead shells. No oysters or oyster shells were observed. Thirty mussels were sampled. Twenty-two were infected by *Marteilia* sp.

2. Mussels were sampled at Haugland, in a shallow, sheltered area between a cluster of small islands. There were patches of mussels on ropes, rocks and hard bottom areas. Here were numerous empty shells, and several year classes of mussels. No oysters were observed. Thirty mussels were sampled for histology. Mussels had empty gonads and low condition. Four were infected by *Marteilia* sp. Digestive gland samples from 50 mussels were analyzed by PCR. Eleven were *Marteilia* positive. There was correspondence between microscopy and PCR.

A Restriction Fragment Length Polymorphism (RFLP) analyse (Le Roux et al. 2001) was performed on positive samples and showed the presence of *M. refringens* Type M / *M. pararefringens*. Sequencing of samples from the RFLP analyse verified the Type M, based on the ITS-sequence. Blast results from sequences from this region give the highest score for *M. refringens* / *M. pararefringens* and sequences have the same signature sequence as *M. pararefringens* (DQ426550) (see Kerr et al. 2018).

Strømmen, Rissa, Trøndelag (63.5776946, 9.940414)

Mussels from Rissastrømmen come from a wide tidal current used as intermediate grow-out and harvest of farmed mussels. Fifty-two mussels were examined by microscopy. Mussels appeared in good but variable condition. Haemic neoplasia was observed in two mussels. RLOs were observed in two mussels. No other abnormal finding was recorded.

Vågstranda (62.6040173, 7.3325886)

Twenty-two mussels were collected at the same site as the oysters (see above). Mussels had a normal condition. Eleven exhibited microcolonies, presumably *Coccomyca parasitica* in connective tissues and gonads. Trematode cysts were observed in connective tissues of 3 mussels. *Marteilia* sp. was not observed.

4 Discussion and conclusions

The health status of flat oysters

Norwegian flat oysters, *O. edulis*, appear free from *Bonamia* spp. Healthy flat oysters – which in the present situation particularly means free from *Bonamia* spp. - is a valuable resource, both for European oyster growers, as well as programs aiming at re-stocking the disappearing, historical wild oyster beds. It is an important task to monitor Norwegian stocks and disseminate the information on their health status in order to obtain a consensus on how to protect and care for this resource. The institute of Marine Research has started a monitoring of Norwegian flat oyster populations and aims at using the data gained in a Nordic and European context. The monitoring of stocks is linked to the national health surveillance, and through the contact with European scientists to both genetic studies (Margen project) and re-stocking programmes (https://noraeurope.eu/). Flat oysters from Hafrsfjord in Rogaland have already been translocated and seeded on hard bottom sites off the coast of The Netherlands.

The wild flat oyster populations examined in 2020 appear 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 develop into disease problems and potentially induce winter mortalities of affected flat oysters as their nutrient uptake and immune defense are knocked out (Mortensen et al. 2013).

Examination of mussels

Marteilia pararefringens has been found in blue mussels, *Mytilus edulis*, in two traditional heliothermic marine oyster lagoons (Aga and Espevik) in western Norway. These lagoons have much higher water temperatures than the ambient fjord water during the summer season, and have been used for breeding oysters since the 1880's. The semi-closed system of the oyster ponds represents a unique opportunity to study the life cycle and parasite progression of *M. pararefringens* in *M. edulis*.

M. pararefringens have never been detected during our health surveillance of mussels from open water farms or wild mussel beds along the coast. There is however one report of *Marteilia* sp. was observed in blue mussels, *Mytilus edulis* used for environmental monitoring around 2010 (Aarab et al. 2011), but this finding was not notified. In 2020, the Norwegian Veterinary Institute reported *Marteilia*-infected mussels from an open-water farm. Sampling of mussels from two near-by wild beds revealed a high prevalence of *M. pararefringens* and heavily infected mussels. These findings indicate that *M. pararefringens* is not restricted to the heliothermic lagoons. This finding represents a new situation, where the parasite occurs outside a closed lagoon environment. In the continuation of this study, we will look more closely at the traditional oyster lagoons, mussels in closed, sheltered areas and try to understand the distribution and possible movements of *M. pararefringens* in the mussel populations. In order to optimize the sampling regime in the surveillance programme, we will collaborate with all active mussel producers in order to map the farms, spat collection areas and translocations and initiate sampling in the vicinity of a number of lagoons.

The Norwegian mussel production is concentrated in Trøndelag and Nordland, where two dispatch centers receive mussels from several producers in a large geographic area. Although two selected sites are included in the surveillance programme, we do not have a proper overview of the production in this area. The dispatch centers will be followed up and their sources of mussels mapped. The situation illustrates the need for combining the surveillance with the health control of shellfish farms.

Based on our findings, *M. pararefringens* does not seem to infect flat oysters. It is however important to be sure that oysters do 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 and recommendations

Revision of the programme. Selection of sites and increased sample size

The surveillance will be continued according to the plan discussed with the Norwegian Food Safety Authority, and with the aim of obtaining a time series of data on the health status of flat oysters and mussels along the Norwegian coast:

The surveillance programme will be focused on mussel and oyster farms and/or populations that are commercially harvested for on-growth, transport to the markets or used in re-stocking programmes.

The detection of *Marteilia pararefringens* in mussels in new areas indicate that this parasite is more widely distributed than previously known. We consider the sample size of 30 specimens (or 20 as applied in the Veterinary health control of commercial farms) as too low. This sample size should only be used if the health status is known, after a previous two-year surveillance based on 150 specimens every 6 months, based on the principles laid down in EU Decision 2015/1554. To improve the screening and ensure a rapid detection, we started using a method based on 100 specimens per sampling, whereas 50 was analyzed by PCR and 50 by histology. We will increase the sample size to 150 in 2021 to ensure full compliance with the new EU regulations which are implemented in 2021.

The health surveillance is linked to monitoring of stocks, oyster genetic studies and European oyster projects, strengthening the scientific basis for a strong and adequate management of the few remaining, healthy flat oyster populations in Europe.

Knowledge on *Marteilia pararefringens* – progress of the work

Flat oysters do not appear susceptible to *M. pararefringens*. Future scientific studies will focus on understanding the infection and life cycle of *M. pararefringens* in mussels, as a background for a risk assessment and an epidemiological study of the spreading of this parasite in North European mussel populations.

Categorization of mussel and flat oyster production areas in Norway

The samples from Trøndelag collected between 2018 and 2020, and the present mussel sample from Vågstranda represent the first study of the health status of mussels north of Bergen. Due to the lack of data on the health status of mussels from most of the Norwegian coast, the categorization should be discussed and potentially revised.

Absence of *Bonamia* spp. in Norway

We consider Norwegian populations of flat oysters as free from *Bonamia* spp. This makes the Norwegian flat oysters a valuable resource, for the oyster producers as well as initiatives of re-stocking the vanishing populations of native European oyster beds.

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