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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 2015

By Stein Mortensen, Lisbeth Sælemyr, Cecilie K. Skår, Torjan Bodvin and Anders Jelmert

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HAVFORSKNINGSINSTITUTTET
INSTITUTE OF MARINE RESEARCH

Contents

Summary

Bonamia ostreae/*B. exitiosa* or *Marteilia* sp.was not detected during the surveillance programme in 2015.

The programme was revised in 2015: We selected four wild beds and one oyster farm, based on the present distribution of wild flat oyster beds, and the structure of the (very limited Norwegian) 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 during the surveillance. In oysters, cells resembling "microcells" were observed as previously, with an approximate prevalence of 10 % in the spring samples from Langestrand, and 50 % in the spring samples from Hui, with a very low intensity, and as individual cells. No inflammation or pathological alterations were observed, and the oysters appeared in good health. A *Bonamia* specific Real-time PCR was performed on all 150 specimens from Langestrand in the spring and 16 specimens from Hui where "microcells" were observed in the spring sample. *Microcytos* specific Real-time PCR and nested PCR were performed on the 16 oysters from Hui, and 18 oysters from Langestrand, where the microcells were observed in the spring sampling. All samples were negative with both assays, and the observed cells are therefore not interpreted as *Bonamia* sp. or *Microcytos* sp. One additional mussel sample was included, after a report of abnormal mortalities in several mussel farms in Åfjorden, Trøndelag. No pathogens or pathological alterations were observed.

Very few flat oysters were observed at Hui in Vestfold, indicating that the populations in this area are low. We recommend excluding this site in 2016, and instead include the oyster spat producer at Aga, Bømlo. This will give a better overview of the producers and a control of spat that are distributed to the farms that are still in operation.

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 Langestrand in 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. Sequencing of the PCR products gave 100% identity with *B. ostreae*. After this diagnose, both the Norwegian Veterinary Institute and The Institute of Marine Research have monitored the population. The observed "microcells" have been observed since the sampling at the site was initiated, always at a low prevalence and intensity. No inflammation, pathology or reductions of the oyster's condition have been associated with the observation. The population appears healthy, with a normal reproductive cycle pattern. Several cohorts have been present throughout the study period. Since 2009, more than 2 200 oysters have been examined by histology, and samples from 581 of the oysters have been analyzed by PCR, all with negative results. The situation has thus been stable since 2006 (see Mortensen *et al*. 2016).

In 2015, the surveillance programme for bonamiosis and marteiliosis in European flat oysters, *Ostrea edulis*, and blue mussels, *Mytilus* sp. was revised. This report briefly gives an overview of the present situation, sampling strategy, results from 2015 and suggestion for the 2016 sampling.

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 in 2015.

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

At Hui, 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 delivered to, or sent to, IMR Bergen by over-night mail (Table 1).

At Ytre Hvaler, Østfold: 60 wild mussels were collected at Stuevikskjæret, east of Skjærhalden, close to the Swedish border in October. This site was selected in order to search for *Marteilia* sp. that has been detected in Swedish mussels, during autumn sampling (A.

Alfjorden, SVA, pers. comm.). Mussels were sent live to the IMR laboratory in Bergen. Imprints were prepared from pieces of digestive gland from 30 mussels. Standard sections for histology were prepared from 30 mussels, as described below.

One additional sample from Åfjorden Sør-Trøndelag, was included, after a report of high mortality in several mussel farms during the late autumn 2015. The farmers reported sudden losses of up to 40 % from suspended cultures of mussels in several farms. At this site, imported mussels from Limfjorden were (illegally) re-seeded in 2014. The farmers therefore requested a test of surviving mussels from this site.

All oysters and mussels were processed at the IMR laboratory in Bergen, according to standard methodology. Briefly; Tissue imprints were stained with Hemacolor. 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.

In addition to cytological and histological examination, the mussels from Åfjorden were observed through a binocular: the intestine was dissected out and observed for the potential presence of the intestinal parasite *Mytilicola intestinalis*.

A Real-time PCR (Marty *et al*. 2006) was performed on all 150 oysters from Langestrand collected during the spring samples, and 16 specimens from Hui collected during the spring, where "microcells" were observed.

After analyzing the samples from spring 2015, and due to the combination of the microcells observed and the negative PCR results, we collected 20 oysters at 25 November, thus after the autumn sampling (Table 1). In order to obtain a higher number of target cells for the observed microcells, approximately 2 ml haemolymph was withdrawn from the adductor muscle of each oyster. Haemocytes were pelleted, DNA isolated and tested for *Bonamia* sp. and *Microcytos mackini* by PCR using the Bo/Boas (Cochennec *et al*. 2000) and BON-319F/BON-524R (Hill *et al*. 2010) primers, and the real-time PCR assays described by Marty *et al*. (2006) and Polinski *et al*. (2015).

Additionally, a *Microcytos mackini* specific real-time PCR (Polinski *et al*. 2015) and a standard mikrocytid PCR assay (Hartikainen *et al*. 2014) was performed on DNA from the 16 oysters from Hui, and 18 oysters from Langestrand, where the microcells were observed in the spring sampling. Positive controls were provided by Gary Meyer at Virginia Institute of Marine Science, USA.

Thereafter, the Veterinary Institute provided new sections cut from the two original paraffin blocks containing tissues from the two *Bonamia* positive oysters from 2008. Ten serial sections from each oyster were processed for histological examination and examined as described above.

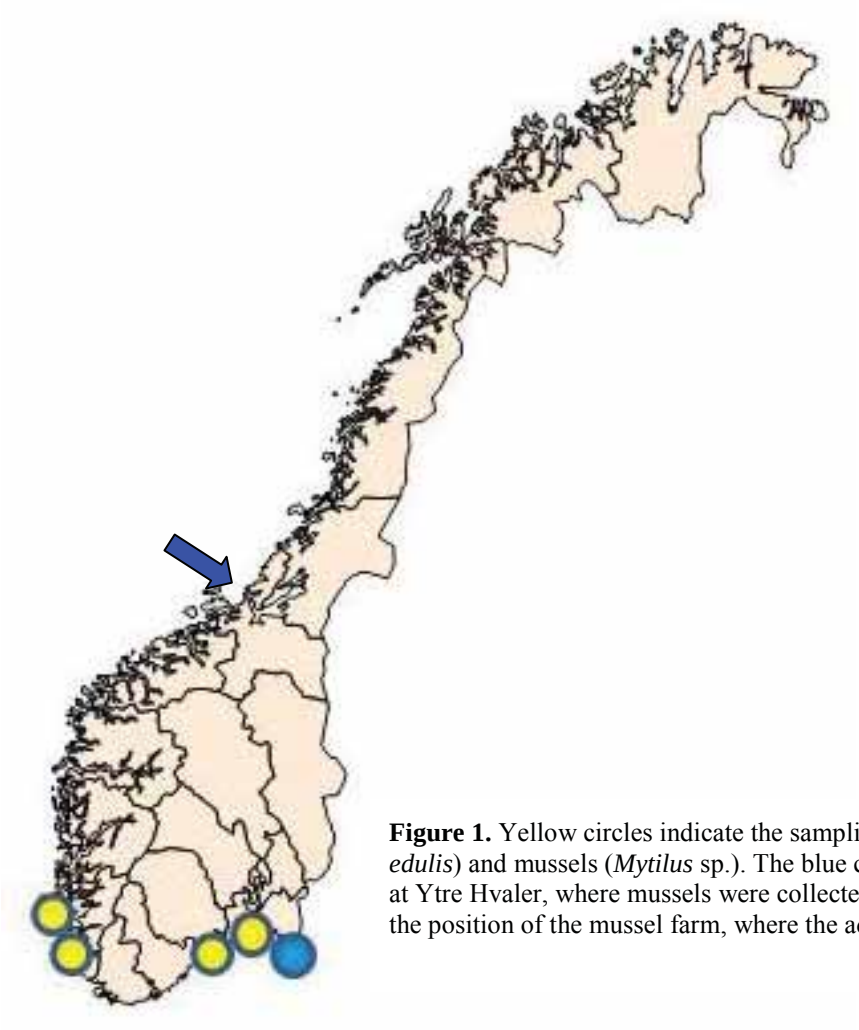

Figure 1. Yellow circles indicate the sampling sites for flat oysters (*Ostrea edulis*) and mussels (*Mytilus* sp.). The blue circle indicates the sampling site at Ytre Hvaler, where mussels were collected in October. The arrow marks the position of the mussel farm, where the additional sample was collected.

Table 1. Sampling and surveillance sites for flat oysters (*Ostrea edulis*) and mussels (*Mytilus* sp.) in 2015.

Sampling site	Oysters		Mussels	
	Spring	Autumn	Autumn	
Ytre Hvaler, Østfold			60	
Hui, Vestfold	30	30	30	
Langestrand, Aust-Agder	150	150 25	30	Extra; PCR on haemocytes
Hafrsfjord, Rogaland	30	30	30	
Sveio, Hordaland	30	30	30	
Åfjorden, Sør-Trøndelag			60	Follow-up on reported abnormal mortality late autumn – winter
Total	240	265	240	

Results

Langestrand, Aust-Agder

The site was inspected by skin diving in May 2015. Dense oyster beds were observed down to approximately 4 m 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.

During examination of the flat oysters, gross morphology of shells and soft parts appeared normal. During microscopical examination, "microcells" were observed as previously, with a prevalence of 10 % and a very low intensity (Figure 2), where cells always appeared individually, and in very low numbers. No inflammation or pathological alterations were observed, and the oysters appeared in good health. Results from the Real-time PCR for *Bonamia* spp. (Marty *et al*. 2006; Hill *et al*. 2010) and *Microcytos* sp. / *Mikrocytos mackini* (Polinski *et al*. 2015; Hartikainen *et al.* 2014) were negative. Positive and negative controls gave expected results.

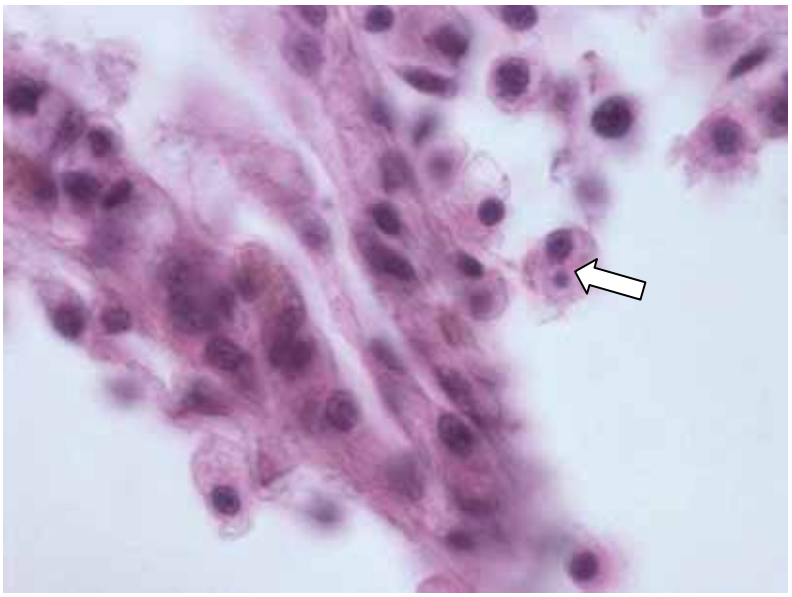

Figure 2. Detail from a gill section from a flat oyster, *Ostrea edulis*, from Langestrand, spring 2015. A free haemocyte with a possible microcell (arrow) (HES staining).

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.

PCR analyses of haemocyte samples from November were negative for both *Bonamia* sp. and *Microcytos mackini*, while positive and negative controls gave expected results.

Mussels collected in October 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.

Examination of archived material from Langestrand 2008

Examination of sections from the paraffin blocks from 2008, were in accordance with all previous samples: cell structures that could represent microcells were observed, but images were not perfectly clear and difficult to interpret. No inflammations or pathological alterations were observed

Hafrsfjord, Rogaland

Two sites (Sola and Sørnes) were inspected by skin diving in May 2015. Samples were collected at Sørnes in May and October (Table 1). At Sola, few flat oysters were observed. At Sørnes, dense, patchy oyster beds were observed down to approximately 3 m 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. 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. *Bonamia* sp. or *Marteilia* sp. were not observed.

Mussels collected in October had a low meat content / poor condition, however no pathogens or abnormalities were observed.

Sveio, Hordaland

Condition index of the oysters was variable in the spring, higher and more uniform in the autumn. Mussels appeared normal. No pathogens or abnormalities were observed.

Hui, Vestfold

The site was inspected by skin diving in May and October. The site was influenced by earlier mortality events during the winters 2009 – 2011, with empty shells and low numbers of flat oysters and mussels. Some shallow water areas were dominated by Pacific oysters.

During microscopical examination of flat oysters, "microcells" were observed, with prevalence of 50 % and a very low intensity, where cells always appeared individually, and in very low numbers. No inflammation or pathological alterations were observed, and the oysters had a variable but normal condition.

Mussels appeared normal, however 8 specimens had green pustules, presumably representing infections with the parasitic algae *C. parasitica*. *Marteilia* sp. was not observed.

Ytre Hvaler, Østfold:

Mussels appeared normal. *Marteilia* sp. was not observed neither in imprints nor histological sections. A few mussels had green pustules, presumably due to *C. parasitica*.

Åfjorden, Sør-Trøndelag

Mytilicola intestinalis was not observed during examination of the intestines. No pathogens or pathological alterations were observed.

Discussion

The observed microcells have been observed in flat oysters since the sampling at the site was initiated, always at a low prevalence and intensity. No inflammation, pathology or reductions of the oyster's condition have been associated with the observation. The population appears healthy, with a normal reproductive cycle pattern. Several cohorts have been present throughout the study period. More than 2 200 oysters have been examined since 2008, and samples from 581 of these have been analyzed by PCR, all with negative results (Mortensen *et al.* 2016). The situation has thus been stable since 2006. A 10 years long sub-clinical *Bonamia* infection seems unlikely, taking into account that this oyster bed experiences extremely variable conditions through the seasons.

The observed microcells are slightly larger than *B. ostreae* compared to reference samples and have a more centric nucleus (Figure 2), resembling *Bonamia exitiosa*. These should however have been detected by the real-time PCR used. Due to the size and central nucleus, the cells could also be interpreted as *Microcytos mackini*. The *M. mackini* PCR however also turned out negative, and the localization of the cells did not correspond to a classical *Microcytos* detection: The observed cells are always detected in haemocytes, in contrast to *Microcytos*, which are normally found in connective and muscular tissues. The haemocyte sampling was however done after the autumn sampling, in which microcells were not observed. The cells might thus have been absent in the last batch of oysters.

This case remains unresolved. If the diagnosis from 2009 is correct, *Bonamia* must be present at a very low prevalence, escaping PCR detection due to the sample sizes in the present study and in co-existence with the oysters, thus not causing disease or killing its host.

One possible explanation is that the observed cells are not closely related to *Bonamia ostreae*, but another organism not detected by the assays used. If there were sufficient DNA present in any of the samples analyzed by PCR, the known *Bonamia* species – including *B. exitiosa* - should have been detected. Other haplosporidian parasites should have been detected by one of the assays applied (Cochennec *et al*. 2000) on the gill samples in 2009 and the haemocyte samples. In this context, the original diagnose remains a mystery.

Conclusion and recommendations

We consider the bivalves examined in 2015 as negative with regard to *Bonamia ostreae* / *B. exitiosa* and *Marteilia refringens*.

A new extraction of DNA from haemocytes from oysters from Langestrand will be performed during spring and summer 2016, when the microcells are presumably present. The results will be reported separately, after the analysis has been performed.

Very few flat oysters were observed at Hui in Vestfold, indicating that the populations in this area are low. We recommend excluding this site in 2016, and instead include the oyster spat

producer at Aga, Bømlo (that was previously included in the programme). This will give a better overview of the producers and a control of spat that are distributed to the farms that are still in operation.

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