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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 2016

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Summary

The surveillance programme was carried out in accordance with the model established in 2015, including 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* or microcells resembling *Bonamia* spp. were not detected during the surveillance programme in 2016.

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. From one mortality event, at Ytre Hvaler, Østfold, South-eastern Norway, in September 2016, live, presumably moribund mussels were sent to IMR and examined. Affected mussels showed infiltration of bacteria in digestive and connective tissues. Bacteria have been isolated, cultured and frozen for subsequent analysis. No parasites were observed.

Marteilia refringens was detected for the first time in mussels, *Mytilus* sp. collected at Bømlo, western Norway in October. The prevalence was 50% and most infected mussels exhibited a heavy infection with sporulating *Marteilia* cells in the digestive epithelia, and affected tissues. A plan for extended survey and a study of the *Marteilia refringens* in affected mussels has been initiated.

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 2 500 oysters have been examined by histology and/or PCR, all with negative results. The situation has thus been stable since 2006 (see 2015 report and Mortensen *et al*. 2016).

The surveillance programme for bonamiosis and marteiliosis in European flat oysters, *Ostrea edulis*, and blue mussels, *Mytilus* sp. was revised in 2015. This report briefly gives an overview of the present situation, results from 2016 and suggestion for the 2017 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 also in 2016.

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 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: an acute mortality occurred in September. Live, presumably moribund mussels were sent to IMR by over-night mail. Imprints were prepared from pieces of digestive gland from 30 mussels. Standard sections for histology were prepared from 30 mussels as described below. Bacteria were collected according to SOP from EURL: Haemolymph was withdrawn from the sinus of the posterior adductor muscle of 5 mussels, without opening the mussels. Haemolymph was diluted in sterile seawater and plated onto Marine agar. Petri dishes were incubated at 20 C for 48-72 hours, harvested and frozen for later analyses.

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

Additional oysters were collected at Langestrand in 2015, and analyzed in 2016. Due to the combination of the microcells observed in 2015 and the negative PCR results, we collected extra haemolymph samples from Langestrand in 2016. We collected 30 samples in May, 20 samples from September and 20 samples in October. 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. real-time PCR as described above (Marty *et al*. 2006 and Corbeil et al 2006).

After the observation of *Marteilia* sp. by histology, DNA was extracted from ethanol fixed mussel (Aga and Ytre Hvaler (see above) and oyster (Aga) digestive gland tissues. The oysters from Bømlo were sampled at the same time and location as the infected mussels. (Table 1) Real-time PCR and traditional PCR were performed on these samples according to the SOP from EURL. *Marteilia refringens* detection and typing was done with by Real time Polymerase Chain Reaction, and as described by Le Roux et al (2001).

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 sites for flat oysters (*Ostrea edulis*) and mussels (*Mytilus* sp.) in 2016.

Sampling site	**Oysters**		**Mussels**	
	Spring	Autumn	Autumn	
Ytre Hvaler, Østfold			60	
Langestrand, Aust-Agder	150 30	148 20 + 20	30	Extra; PCR on haemocytes
Hafrsfjord, Rogaland	30	30	30	
Sveio, Hordaland	30	30	30	
Aga, Bømlo, Hordaland	30	30	30	

Results

Langestrand, Aust-Agder
The site was inspected by skin diving in May 2016. 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. *Bonamia ostreae* / *B. exitiosa* or microcells resembling *Bonamia* spp. were not detected.

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 analysis of haemocyte samples from May, September and October were negative for *Bonamia* sp. while positive and negative controls gave expected results.

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 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.

Sveio, Hordaland
No pathogens or abnormalities were observed.

Aga, Bømlo, Hordaland
Condition index of the oysters was low. No pathogens were observed by histology. *Marteilia* sp. was detected for the first time in mussels, *Mytilus* sp. collected at Bømlo, western Norway in October. The prevalence was 50% and most infected mussels exhibited a heavy infection with sporulating *Marteilia* cells in the digestive epithelia, and affected tissues. Two specimens had low infections of *Marteilia* in primary stage in digestive duct epithelia.

PCR and sequencing verified that this was *Marteilia refrin*gens type M. (Le Roux et al 2001). There were 16 positive samples positive among the mussels. *Marteilia* was not observed by histology in the oysters, but 10 of 30 oyster samples were positive by PCR. Sequencing verified that it was the same type as in the mussels (*M. refrin*gens type M). Histological slides as well as tissue samples were sent to EURL (IFREMER, France). The diagnose was verified. OIE and EU have been notified. A plan for extended survey and a study of the *Marteilia refringens* in affected mussels has been initiated, and the first additional samples were collected in April 2017.

Ytre Hvaler, Østfold
Moribund mussels showed infiltration of bacteria in digestive and connective tissues. Bacteria have been isolated, cultured and frozen for subsequent analysis. No parasites were observed. *Marteilia* PCR was negative.

Discussion and conclusions

The flat oyster populations examined appears healthy, with a normal reproductive cycle pattern. 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. An 11 years long sub-clinical *Bonamia* infection seems unlikely, taking into account that this oyster bed experiences extremely variable conditions through the seasons. All extra samples of haemocytes taken in May, September and October and analyzed with PCR were *Bonamia* negative. These analyses should have increased the sensitivity of the diagnostic survey significantly: The samples constituted several million haemocytes from each oyster, and haemocytes are target cells for *Bonamia* spp. *In situ* hybridization testing of paraffin embedded tissues are often used as a confirmatory test, in addition to PCR. An *in situ* hybridization testing of paraffin embedded tissues from oysters sampled in 2015 (see 2016 report) is in progress. We consider the bivalves examined in 2016 as negative with regard to *Bonamia ostreae* / *B. exitiosa*. Due to the fact that the situation is stable (and negative) we suggest that autumn sampling is reduced to 30 or 50 oysters.

The detection of *Marteilia refringens* in mussels collected at Aga in October 2016 was surprising. Samples collected by the Norwegian Veterinary Institute in 2014 were negative. This means that *Marteilia* may have been overlooked, were absent (introduced after 2014 or samples were collected during a period where the parasite was not present in the target tissues).Samples from 2014 should be re-examined and the sampling date used in an analysis of the parasite presumed life cycle and sporulation in the mussels. The oysters collected in the poll, close to the mussels, were histology negative but PCR positive, and sequencing showed that also the oysters had *M. refringens* type M. The positive PCR signals may be due to the presence of *Marteilia* spores released from the mussels. The role of oysters as potential vectors has to be investigated.

The sampling point is an oyster poll. When following up the case, we will go through all movements of oyster spat in and out of the site, in order to design an extended sampling scheme. This will be followed up with an extended survey of wild and farmed mussels and a study of the life cycle, potential intermediate hosts in the poll fauna, in particular zooplankton during the summer season. The plan for the extended survey as well as a potential revision of the surveillance programme will be discussed with the competent authorities.

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