



SUMMARIZING THE SCREENING FOR *BONAMIA OSTREAE* IN NORWEGIAN POPULATIONS OF FLAT OYSTERS, *OSTREA EDULIS*.

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Sammendrag (norsk):

Surveillance and studies performed by The Norwegian Institute of Marine Research and the Norwegian Veterinary Institute has never revealed notifiable diseases in Norwegian populations of European flat oysters, *Ostrea edulis*. 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. The cells were however not interpreted as *B. ostreae* and PCR-analysis of samples from this oyster population were negative. In 2008, the EU reference laboratory (EURL) received samples from the Norwegian Veterinary Institute and reported one *Bonamia* sp. in a haemocyte from one oyster, based on microscopy. 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 results are briefly reported here. The observed microcells were found in most samples 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 year classes have been present throughout the study period. Since 2009, more than 3 000 oysters have been examined. *Bonamia* sp. has never been detected, and all PCR assays have been negative.

Sammendrag (engelsk):

Overvåking og studier gjennomført ved Havforskningsinstituttet og Veterinærinstituttet har aldri avdekket meldepliktig sykdom i norske flatøsters, *Ostrea edulis*. I 2006 ble det observert mikroskopiske celler som liknet parasitten *Bonamia ostreae* i østers som var samlet inn i forbindelse med helseovervåking. Cellene ble imidlertid ikke tolket som *B. ostreae* og PCR-analyser gjort av østers fra denne lokaliteten var negative. Skjellene kom fra en vill bestand nær Arendal i Agder. I 2008 sendte Veterinærinstituttet prøver til EUs referanselaboratorium (EURL). Ved EURL observerte man ved mikroskopering en *Bonamia*-celle i en østers. Ved real-time PCR-analyse fikk de positivt signal fra to østers (en av triplikate prøver). Sekvensering ga match med *Bonamia ostreae*. Etter denne diagnosen har både Veterinærinstituttet og Havforskningsinstituttet overvåket bestanden. Resultatene fra dette arbeidet er summert i rapporten. De mikroskopiske cellene er jevnlig observert i prøver fra denne lokaliteten, men alltid med lav prevalens og intensitet. Det er ikke observert inflammasjon eller patologi forbundet med funnene. Østersen er i god kondisjon, bestanden reproducerer som normalt og det er ikke observert tegn til forhøyet dødelighet. Siden 2009 er det analysert mer enn 3 000 østers fra denne lokaliteten. *Bonamia* sp. er ikke påvist, verken ved histologi eller PCR.

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1 - Background

The European flat oyster is indigenous to Norwegian coastal waters. Its distribution and abundance is limited by climatic conditions, and has varied considerably in modern time. There are relatively good records dating back to the 1800's. Oyster farming in Norway was established in 1879 and had its "golden age" until the 1930's. In this period, oysters were moved and exchanged between some of the Norwegian, Danish and Dutch farm and cultivation sites. There have been no known introductions after this period. The present farming of flat oysters is limited to a few farms, and there is a small-scale commercial harvest from wild stocks. Some wild Norwegian populations are presumably naïve. The Institute of Marine Research monitor the stocks and carries out a health surveillance programme.

2 - Health surveillance of Norwegian oysters

The first health surveillance of farmed bivalve mollusks in Norway was carried out between 1989 and 1992 as a part of a research project (Mortensen 1993) and included flat oysters from two of the main oyster farming sites (Espevik and Vågstranda). *Bonamia* sp. was not detected in this study.

In 1995, we did a thorough mapping of the remaining oyster farms and farm sites with viable populations. We also mapped some of the oyster beds in southern and western Norway. The results were used as a background for the first official surveillance program for the notifiable oyster parasites *Bonamia* sp. and *Marteilia refringens* in Norwegian flat oysters, initiated by the Norwegian Food Safety Authority in 1995. The surveillance included several oyster farms, as well as wild beds. There has been a close contact between the oyster farmers and personnel running the surveillance program in order to ensure that any abnormal mortality was reported. No abnormal mortality has been reported, and neither *Bonamia* sp. nor *Marteilia refringens* have been detected in the flat oysters. In 2004, Norway was officially declared a *Bonamia* and *Marteilia* free zone, based on historical data.

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 from 2016, also including blue mussels. 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 are present (Engelsma et al. 2010; A. Alfjorden pers.comm). *Bonamia* spp. has never been observed. Possible microcells were observed in oysters from two sites (see below). *Marteilia refringens* Type M / *M. pararefringens* was detected in mussels, *Mytilus* sp. for the first time in 2016.

3 - The *Bonamia ostreae* diagnose in 2008 – 2009

In 2006, cells resembling *Bonamia* sp. were observed during histopathological examination of tissue specimens of flat oysters, *Ostrea edulis* from the Arendal area, southern Norway. The cells were slightly larger than *B. ostreae* compared to reference samples and had a more centric nucleus (Figure 1). These cells were not interpreted as *Bonamia* during diagnostic surveys, but as they were interpreted as potential “microcells”, samples were sent from the National Veterinary Institute to the European Community Reference Laboratory for Molluscan Diseases in France, for external examination. *Bonamia* sp. was not confirmed. Similar observations were made in 2008, and nine new samples were sent to France. This time the reference laboratory reported one *Bonamia* sp. in a haemocyte from one oyster examined by microscopy. *In situ* hybridization tests (Cochenec *et al.* . 2000) were negative. 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* .

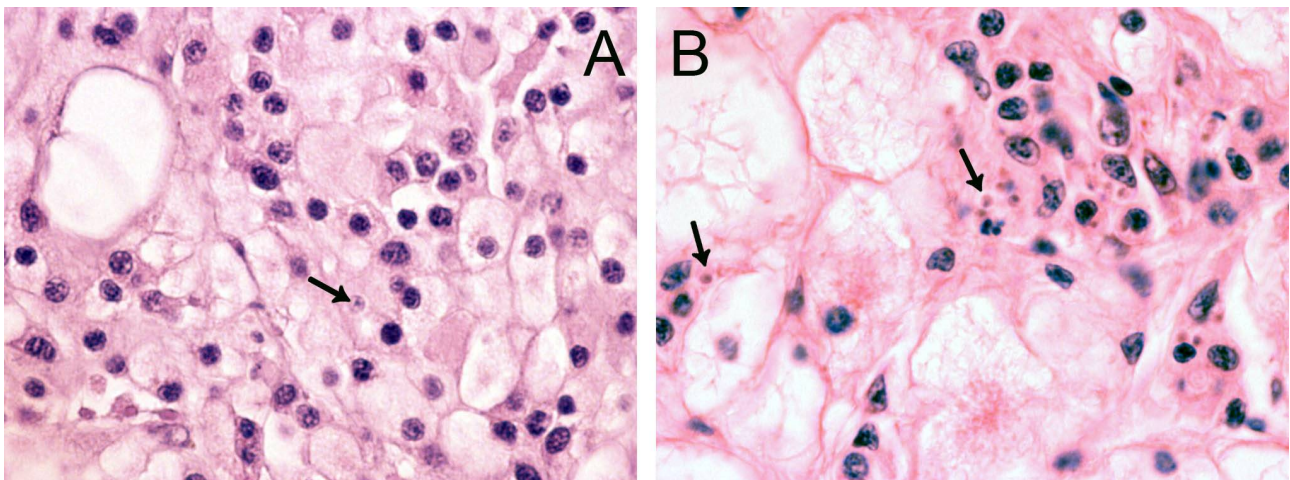


Figure 1. A: Section showing connective/storage tissues from a flat oyster, *Ostrea edulis* from Langestrand near Arendal, June 2009. Numerous haemocytes are present in the tissue. An un-identified cell is marked with an arrow. B: Same tissue area in a reference section from a flat oyster infected with *Bonamia ostreae* , provided by EURL. Several *B. ostreae* are visible (arrows), both freely and inside haemocytes. Both images have the same magnification.

4 - A targeted study in 2009

After the positive diagnosis, a study was initiated by the Food Safety Authority (Mattilsynet) in June 2009, to follow up the case. A new investigation was performed on the oyster population by the Institute of Marine Research (IMR), as well as at three neighboring populations, in order to try to confirm the diagnosis, determine the potential spreading of the parasite and compare histology and PCR as diagnostic tools. Oysters from a farm at Bømlo, on the Norwegian west coast, were used as a presumably negative control. The sites were inspected by skin-diving. There were no signs of recent or previous mass oyster mortalities that could be related to bonamiosis at the examined sites. 50 oysters from each of the four sites were collected and subjected to standard histopathological examination and PCR, using the BO/BOAS (Cochennec *et al.* 2000) and Cf/Cr (Carnegie *et al.* 2000) primers. Microscopy of the collected samples confirmed the histopathological findings observed throughout the previous years. However, *Bonamia*-like cells coinciding with an infection pattern as described for this parasite were not observed: The microcells were present at a very low prevalence, and when present, always as a few single cells. No propagation, inflammation or pathological changes were observed. The PCRs gave negative or inconclusive results, using the two different assays. The investigations did thus not confirm the *Bonamia* diagnose.

5 - Surveillance and studies after 2009

The official surveillance program has been continued after 2009, carried out by the Veterinary Institute until 2014, with an increased number of oysters collected from the same site every six months (however with some samplings including less than the required 150 oysters (Table 1, data from Veterinary Institute annual reports from the surveillance programme). *Bonamia* sp. was not confirmed. In parallel to the surveillance program, monitoring of the population, histological examinations and PCR analysis were carried out by the Institute of Marine Research (included in Table 1). From 2015, the surveillance and control programme has been carried out by IMR.

Table 1. Surveillance, microscopy and analyses of flat oyster samples from the population at Langestrand in Agder County in the period 2009- 2019, carried out at the Veterinary Institute (VI) and Institute of Marine Research (IMR).

2009	Spring/summer	VI	146 oysters	Histology
		IMR	200 oysters	Histology PCR (Marty <i>et al.</i> 2006)
	Autumn	VI	112	Histology
2010	Spring	VI	144	Histology
	Summer	IMR	50	Histology PCR (Marty <i>et al.</i> 2006 & Corbeil <i>et al.</i> 2006)
	Autumn	VI	123	Histology
2011	Spring	VI	150	Histology
	Summer	IMR	50	Histology PCR (Marty <i>et al.</i> 2006 & Corbeil <i>et al.</i> 2006)
	Autumn	VI	150	Histology
2012	Spring	VI	100	Histology
	Summer	IMR	50	Histology PCR (Marty <i>et al.</i> 2006 & Corbeil <i>et al.</i> 2006)
	Autumn	VI	0	Histology
2013	Spring	VI	98	Histology
	Summer	IMR	50	Histology PCR (Marty <i>et al.</i> 2006 & Corbeil <i>et al.</i> 2006)
	Autumn	VI	145	Histology
2014	Spring	VI	150	Histology
	Summer	IMR	19	Histology PCR (Marty <i>et al.</i> 2006 & Corbeil <i>et al.</i> 2006)
	Autumn	VI	147	Histology
2015	spring	IMR	150	Histology PCR (Marty <i>et al.</i> 2006)
	autumn		150	Histology
	winter		20	PCR on haemocytes (Marty <i>et al.</i> 2006)
2016	spring		150 30	PCR on haemocytes
	autumn		148 20+20	PCR on haemocytes
2017	spring		150 30	
	autumn		30	
2018	Spring		150 (20) 30	Not yet examined. Histo & PCR (30) spring
	autumn		30	
2019	spring		150	Not yet examined.
Total			3042	

6 - Surveillance in 2015 and 2016 included targeted PCR on haemocytes

In May 2015, we re-examined the site. There was no sign of abnormal mortality, and several age groups present. 150 flat oysters were collected, processed by standard methods and examined by histology. 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. Real-time PCR (Marty *et al.* 2006) was performed on all 150 specimens. All samples were negative, while positive and negative controls gave expected results. The sampling of 150 oysters was repeated in October. There was no sign of abnormal mortality. Microcells were not observed during the histological examination. The oysters appeared in good health. 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. by PCR using the Bo/Boas (Cochenec *et al.* 2000) and BON-319F/BON-524R (Hill *et al.* 2010) primers, and the real-time PCR as described above (Marty *et al.* 2006). All samples were negative, while positive and negative controls gave expected results.

Due to the combination of the microcells observed in 2015 and the negative PCR results, we collected extra haemolymph samples from Langestrand (Agder, see fig. 3) in 2016. We collected 30 samples in May, 20 samples in 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). All samples were negative.

Additionally, a *Microcytos* sp. real-time PCR (Polinski *et al.* 2015) was performed on DNA from the haemocyte samples. Positive controls were kindly provided by Gary Meyer at Virginia Institute of Marine Science. All samples were negative, while positive and negative controls gave expected results.

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 examined at IMR. Observations 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.

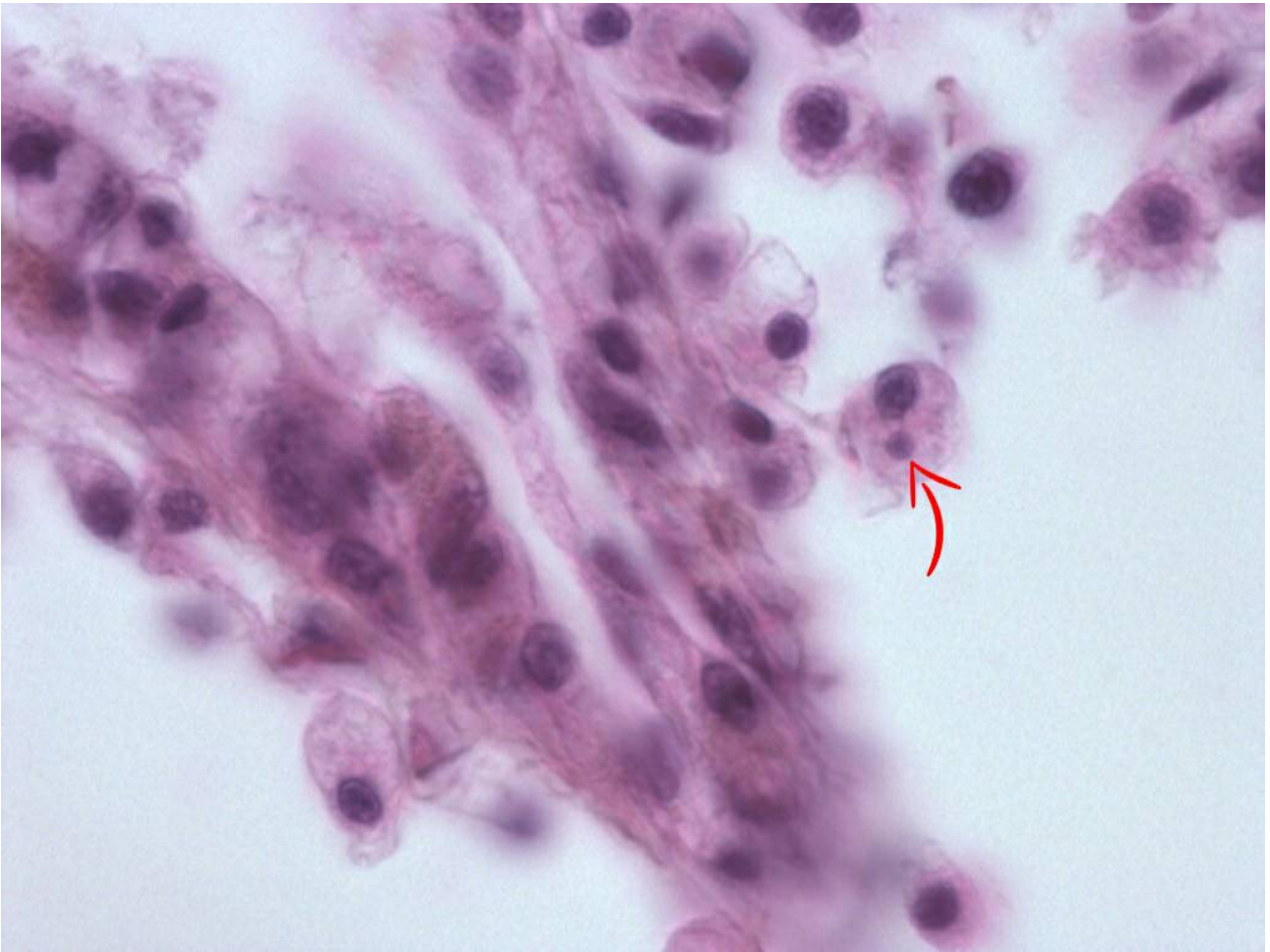


Figure 2. Detail from a gill section from a flat oyster, *Ostrea edulis*, from Langestrand, spring 2015. A free haemocyte with a possible microcell (arrow) (HES staining).

7 - Examination of oyster samples from other sites

In addition to the samples from Langestrand, IMR has followed some of the oyster production sites more or less regularly since 1989. Much of the work has focused on the health of flat oysters in some of the farming units producing oyster seed. *Bonamia* spp. has never been observed. Some of the results from the health surveillance is not published. However, several sites were included in the Surveillance programme for bonamiosis and marteiliosis (National Veterinary Institute until 2014 and IMR since 2015). *Bonamia* spp. has never been detected.

Samples collected and analyzed by IMR during the Surveillance programme (2015 – present) are listed in Table 2, below.

Table 2. Samples from flat oysters, O. edulis collected during the Surveillance programme for bonamiosis and marteiliosis since 2015 and analyzed for the presence of Bonamia spp. Hui is a wild bed near Tønsberg, outer Oslofjord. Hafrsfjord is a wild bed near Stavanger. Sveio is an oyster farm on the west coast. Aga is a lagoon producing oyster spat on the Island of Bømlo, West coast. Innerøyen is a lagoon producing oyster spat near Halhjem, south of Bergen (see Figure 3). Bonamia spp. was not detected in any sample.

Site	2015		2016		2017		2018		2019	
	spring	autumn	spring	autumn	spring	autumn	spring	autumn	spring	autumn
Hui	30	30								
Hafrsfjord	30	30	30	30	30	30	30	30	30	
Sveio	30	30	30	30	30	30	30			
Aga			30	30	30	30	30	30	30	30
Innerøyen									30	



Figure 3. Sampling of flat oysters, *Ostrea edulis*, and blue mussels, *Mytilus* spp. 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.

8 - Discussion

The shoreline and shallow water area at Langestrand inhabit one of the densest flat oyster populations found in Norway. It is presumed to be naïve, and there are no oyster farms in the area. In order to protect this population, it has been protected from commercial as well as recreational harvest since 2012. IMR has monitored the stock and collected samples for histopathological examination every spring since 2012. No sign of diseases affecting the health of the oysters have been observed. The population appears healthy, with a normal reproductive cycle pattern. Several cohorts have been present throughout the study period.

However, structures resembling microcells have been observed since the sampling at the site at Langestrand 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 microcells were never interpreted as *Bonamia* sp. and the diagnose made by EURL was surprising.

Since 2009, more than 3 000 oysters have been examined, and samples from more than 600 of these have been analyzed by PCR, all with negative results. 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 (Figures 1 & 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.

Samples with the observed microcells were also included in a study of microbiota in flat oysters from Norwegian sites, as a part of the VIVALDI project WP1 (<https://cordis.europa.eu/project/id/678589>). PCR assays for *Bonamia* spp, mikrocytids, and paramyxids were all negative. A metagenomic shotgun sequence library was produced from the single available sample, but no candidate parasite sequences were detected. The only positive PCR assay was for haplosporidia, and the products were sequenced. Preliminary phylogenetic analyses showed this to be an early branching haplosporidian. Further work is currently being undertaken to determine whether this sequence derives from the observed microcell.

The nature of the observed cells is thus unresolved. The cells are not believed to be closely related to *Bonamia ostreae* . 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 (Cochenec *et al* . 2000) on the gill samples in 2009 and the haemocyte samples. In this context, the original diagnose remains a mystery. The haplosporidian sequence generated as described above is currently being further investigated.

9 - Acknowledgements

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