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Summary

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. 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. A 10 years long sub-clinical *Bonamia* infection seems unlikely. 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 living in co-existence with the oysters, thus not 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. We will perform a new extraction of DNA from haemocytes during spring and summer 2016, when the microcells are presumably present. As the situation has been unchanged for 10 years, there is no need to sample 150 oysters every six months. The sample size may be reduced to 60. We recommend however restrictions on the movement of bivalve into, and out of, this area, until results from the sampling in spring 2016 has been analyzed and reported.

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. The present farming of flat oysters is limited to a few farms, and there is no commercial harvest from wild stocks. Some wild Norwegian populations are presumably naïve.

Health surveillance of Norwegian oysters

The first health surveillance of farmed bivalve mollusks in Norway was carried out between 1989 and 1992 (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 tried to map 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 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 2004, Norway was officially declared a *Bonamia* and *Marteilia* free zone.

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 (Cochennec *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*.

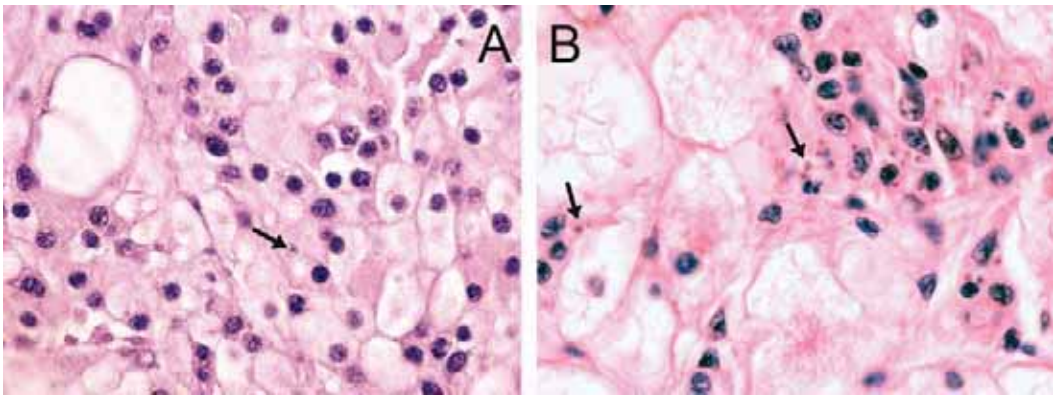

Figure 1. A: Section showing connective/storage tissues from a flat oyster, *Ostrea edulis* from Langestrand near Arendal, june 2009. Numerous haemocytes are present in the tissue. An un-identified cell is marked with an arrow. **B:** Same tissue area in a reference section from a flat oyster infected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

Studies after 2009

After the positive diagnosis, a study was initiated by the Food Authorities (Mattilsynet) in June 2009, to follow up the case. A new investigation was performed on the oyster population, 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.

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)). *Bonamia* sp. has not been confirmed. In parallel to the surveillance program, monitoring of the population, histological examinations and PCR analysis have been carried out by the Institute of Marine Research (included in Table 1).

Table 1. Surveillance, microscopy and analyses of flat oyster samples from the population at Langestrand in Aust Agder County in the period 2009-2015, 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 *et al.* 2006)	On 192: negative
	Autumn	VI	112	Histology	
2010	Spring	VI	144	Histology	
	Summer	IMR	50	Histology	negative
				PCR (Marty *et al.* 2006 & Corbeil *et al.* 2006)	
	Autumn	VI	123	Histology	
2011	Spring	VI	150	Histology	
	Summer	IMR	50	Histology	negative
				PCR (Marty *et al.* 2006 & Corbeil *et al.* 2006)	
	Autumn	VI	150	Histology	
2012	Spring	VI	100	Histology	
	Summer	IMR	50	Histology	negative
				PCR (Marty *et al.* 2006 & Corbeil *et al.* 2006)	
	Autumn	VI	0	Histology	
2013	Spring	VI	98	Histology	
	Summer	IMR	50	Histology	negative
				PCR (Marty *et al.* 2006 & Corbeil *et al.* 2006)	
	Autumn	VI	145	Histology	
2014	Spring	VI	150	Histology	
	Summer	IMR	19	Histology	negative
				PCR (Marty *et al.* 2006 & Corbeil *et al.* 2006)	
	Autumn	VI	147	Histology	
2015	spring	IMR	150	Histology	
				PCR (Marty *et al.* 2006)	negative
	autumn	IMR	150	Histology	
	winter		20	PCR on haemocytes (Marty *et al.* 2006)	negative
Total			**2204**		

Examination of oyster samples in 2015

In May 2015, we re-examined the site. There was no sign of abnormal mortality, and several cohorts 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.

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. PCR analyses have so far not been performed on the autumn samples (from October 2015).

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 (Cochennec *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.

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

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 examined. 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.

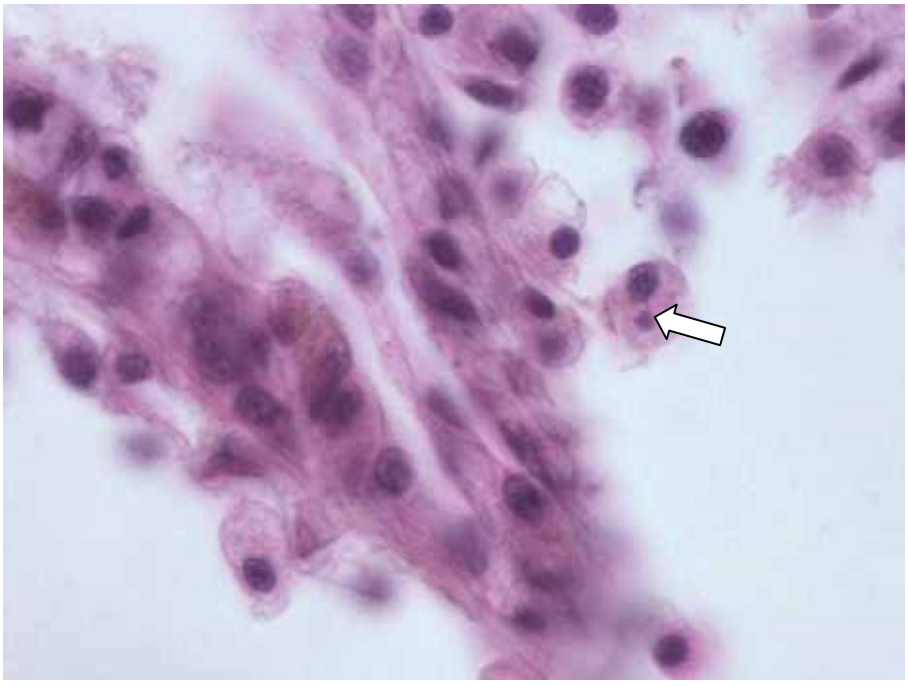

Figure 2. Detail from a gill section from a flat oyster, *Ostrea edulis*, from Langestrand, spring 2015. A free haemocyte with a possible microcell (arrow) (HES staining).

Discussion

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. More than 2 200 oysters have been examined, and samples from 581 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. 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.

We will perform a new extraction of DNA from haemocytes during spring and summer 2016, when the microcells are presumably present. As the situation has been unchanged for 10 years, there is no need to sample 150 oysters every six months. The sample size may be reduced to 60. We recommend however restrictions on the movement of bivalve into, and out of, this area, until results from the sampling in spring 2016 are reported.

Acknowledgements

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