Risk of introduction of *Bonamia ostreae* infected *Ostrea edulis* in the North Sea

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Risk of introduction of *Bonamia ostreae* infected *Ostrea edulis* in the North Sea

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Samenvatting NL. De gewone oester (*Ostrea edulis*) is de inheemse oestersoort in West Europa. Over de afgelopen jaren is de interesse toegenomen in herstelprojecten van de historische oester populatie in de Noordzee. Als platte oesters in de Noordzee worden uitgezet is er, afhankelijk van de oorsprong van de oesters, risico op introductie van de platte oester parasiet *Bonamia ostreae*. Dit rapport geeft een beoordeling van het risico van insleep van *B. ostreae* bij het vrijgeven van partijen oesters wanneer deze partijen vooraf worden getest.
De grootte van het monster die van de partij oesters moet worden getest is afhankelijk van het gewenste betrouwbaarheidsniveau en de veronderstelde prevalentie van de bron. Het herhaaldelijk vrijgeven van partijen geeft een toename in het risico van insleep van ziekte, ook als er met een hoge betrouwbaarheid getest wordt op afwezigheid van de parasiet. Het herhaaldelijk testen van een partij met een quarantaineperiode ertussen (onder de aanname dat er geen veranderingen in de infectiestatus zijn) verhoogt de gevoeligheid van de procedure niet.

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Summary

The European flat oyster (*Ostrea edulis*) is the native oyster species in Western Europe. In recent years there has been interest in restoration of the flat oyster population in the North Sea. When flat oysters are introduced into the North Sea from other sources there is a risk of introduction of the flat oyster parasite *Bonamia ostreae*. This report gives an assessment of the risk of introduction of *Bonamia ostreae* with the release of batches of oysters when the batches are pre-tested before release.
The samples size to be tested from the batch of oysters to assess freedom of disease is depending on the desired confidence level and the assumed source-prevalence. With repeated releases of batches the risk of introduction increases, also when testing for freedom of disease with a high confidence level. Repeated testing of a batch with a quarantine period in between (under the assumption that no changes in infection status) does not increase the sensitivity of the procedure.

1 Introduction

Ostrea edulis (EN: European flat oyster, NL: Platte oesters) is the native oyster species in Western Europe. In the last century the European flat oyster population has decreased considerably due to habitat loss, anthropogenic influences and disease (see Thurstan *et al*. 2024 for historical distribution). In recent years there have been multiple initiatives on flat oyster restoration projects, among which several projects for restoration of the flat oyster population in the North Sea.

Bonamia ostreae is an intracellular parasite infecting the haemocytes of oysters. After introduction in Europe in 1979 it has spread to the main flat oyster culture areas in Europe and is considered endemic in most of these areas. In the Netherlands the parasite was introduced in the Oosterschelde in 1980 (Van Banning, 1982) and has now established itself in the Dutch oyster culture areas (Engelsma *et al*. 2010). From the annual monitoring of mollusc diseases the prevalence of *B. ostreae* in adult oysters from Lake Grevelingen is estimated by real time PCR (Engelsma & Allaart, 2024). The average prevalence in spring (April) over the period 2020-2024 was 12.4%.

In the Dutch part of the North Sea there are no specific disease control measures applied for *B. ostreae* in the context of EU animal health regulation EU/2016/429. However, there is a general commitment to keep this population free from the flat oyster parasite *Bonamia ostreae* (Pogoda *et al*. 2019).

When European flat oysters are introduced into the North Sea in the context of a restoration programme, depending on the source of the oysters, there is a risk of introduction of *B. ostreae*. The Ministry of Agriculture, Fisheries, Food Security and Nature (LVVN) has requested Wageningen Bioveterinary Research (WBVR) to assess the risk of introduction of *B. ostreae* infected European flat oysters in the North Sea under different scenarios with oyster batches pre-tested before release.

For diagnostic testing for freedom of disease, in this case for freedom of *B. ostreae*, real time PCR assays are frequently used and fit for purpose. For *B. ostreae* the real time PCR assay described by the EU Reference Laboratory for Mollusc Disease (IFREMER, La Tremblade, France) is the preferred method to be used for diagnostics on request of EU competent authorities (EURL SOP, 2023). In addition to this assay a second real time PCR assay, published by Marty and coauthors (2006) has been in use for diagnostics at WBVR. In practice the latter assay has shown to be slightly be more sensitive in detection compared to the EURL SOP. On the other hand, this assay is less specific: it does not discriminate between different *Bonamia* species. The characteristics of both assays are used below to assess the risk of introduction using these assays for batch testing.

The aim of this report is to give a better insight in the risks of introducing *B. ostreae* infected oysters in the North Sea with batch test-and-release of flat oysters, assuming the *Bonamia* status of the batch is unknown. The report describes the methods of the assessment, results and analyses the performance of different testing strategies. The risk of establishment and spread after the introduction is out of scope of this assessment.

2 Methods

2.1 Parameters

The risk of introduction of the pathogen *B. ostreae* into an area, depends on several input parameters:

1) the source prevalence
2) the test performance
3) the testing regime (sampling and potential re-testing)

2.1.1 Source prevalence

For the purpose of this study we consider different source prevalences of *B. ostreae*: 1, 2 and 4%. The prevalence of *B. ostreae* in the wild oyster population in Lake Grevelingen can be higher (see introduction). However, this is less relevant for this assessment as these levels will be very likely to be detected during batch testing.

2.1.2 Test performance

Testing of batches of oysters (adults, spat or larvae) is performed using the EU reference lab real time PCR ('EURL PCR') test (eurl-mollusc SOP). As many diagnostic methods, the EURL PCR test is imperfect. This means that it can fail to detect infected oysters (false negatives) or incorrectly labels uninfected oysters as positive (false positives). False negatives are a concern here, since undetected infection poses a risk of infection, and thus a batch falsely gets labelled as free from disease. On the other hand, false positive results might lead to unnecessarily discarding a batch, which does not result in introduction.

In practice, the real time PCR test of Marty et al. (2006; 'Marty PCR') has shown to be a more sensitive test compared to the EURL PCR. For the purpose of this study, this test is considered to be the gold standard. The positive status of the Marty PCR reflects most likely better the "true" positive status. Given the absence of related *Bonamia* species in the source area of the evaluation, the lower specificity of the assay is not a concern for this assessment.

A comparison of the test performance of the EURL PCR compared with Marty PCR is given in Table 1. This consists of oyster samples tested in the context of a mollusc health surveillance in Lake Grevelingen, the Netherlands and used for validation of both PCR assays. Out of the 107 samples that tested positive using the Marty PCR test, 80 tested positive in the EURL PCR, leading to a sensitivity of 80/107 = 74.7%. The probability of a false negative test, given infection is thus 0.253. Additionally, we compare this sensitivity with a hypothetical test with a specificity of 95%.

Table 1. Cross tabulation of test performance: EURL PCR compared to Marty PCR.

Field samples		**Marty PCR**		
		Positive	Negative	**Total**
EURL PCR	Positive	80	0	80
	Negative	27	200	227
	Total	107	200	**307**

2.1.3 Testing regime

The oyster batches to be released could consist of adult oysters, spat or larvae. For a single test 25-50 mg of oyster tissue is necessary. Smaller spat (< 6 mm) and larvae can be pooled for the test to reach this quantity. We assume that using the same tissue quantity the test sensitivity will be maintained, irrespective of the life stage.

We consider two scenarios: 1) Where a batch is tested once, and 2) where a batch is retested after a certain period of time. We assume that between the first test and the second test, both the prevalence of disease and the characteristics of the test remain the same. With this assumption the length of this "quarantine" period does not have influence on the final results. However, due to the sacrifice of animals for testing, the batch size is reduced.

2.2 Calculations

We apply a one-stage sampling scheme for a finite population using an imperfect diagnostic test (Cameron and Baldock, 1998a). This means that we consider a group of animals/batch (with batch size *N*), and we select a certain number of animals at random (with sample size *n*). Subsequently, these animals are tested in order to determine if the entire batch is infected with the parasite or not. This means that we test *n* individuals from a population with size $N \geq n$. If all tested individuals have a negative test result we classify the batch as being free from the disease. If we find one or more individuals that test positive, we classify the batch as diseased. For this test design, we are interested in the probability of correctly classifying the population as diseased. To compute this probability, we need to know:

- the population/batch size *N*,
- the sample size *n*,
- the prevalence π of the disease in the population/batch (or the number of diseased individuals in the population $d = N \cdot \pi$) and
- the sensitivity Se and the specificity Sp of the diagnostic test.

The probability of correctly classifying the batch as diseased is complementary to the probability (P) of finding no test positives ($T^+ = 0$), given that at least *d* individuals are diseased in the population (denoted by $P(T^+ = 0|d)$), which can be computed using a modified hypergeometric formula described in detail by Cameron and Baldock (1998a). Given that the specificity of the test is here assumed to be perfect we use the following formula:

$$P(T^+ = 0 \mid d) = \sum_{y=\max(0,n-N+d)}^{\min(d,n)} \frac{\binom{d}{y}\binom{N-d}{n-y}}{\binom{N}{n}} (1 - \mathrm{Se})^y$$

The formula above describes the probability of failing to detect the parasite, which can be considered the risk of introduction of disease when a batch has tested negative, despite the presence of the parasite.
We often define a threshold of 'acceptable risk' (α) to calculate a sample size for testing for freedom of disease. The optimal sample size is the number of animals that still satisfies $P(T^+ = 0|d) \leq \alpha$, where 1-α can be defined as the 'confidence level'.
We compare batch sizes (*N*) of 1000, 10,000 and 100,000.

2.3 Re-testing

For the scenario re-testing, we consider that the batch size during retesting (*N2*) is reduced with the initial sample size. The prevalence and the test characteristics remain the same. The final probability of failing to detect the disease is calculated as the product of the probability of failing to detect during the first and the second test.

3 Results

3.1 Probability of detection

Figure 1 shows the relationship between the probability that an infection is not detected given that a batch has a certain prevalence of *B. ostreae*. The probability is mainly dependent on the prevalence and sample size. A lower prevalence is more difficult to detect. A similar relationship exists with the sample size: the bigger the sample size, the lower the chance that the disease is undetected. However, batch size (as shown as different line-style in the figure) does not greatly influence the probability.

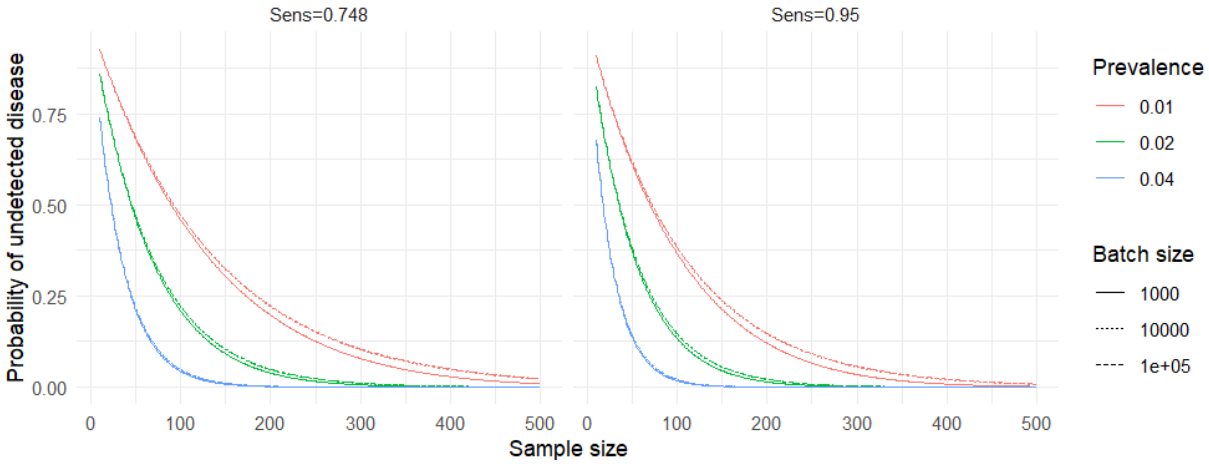

Figure 1. Relationship between probability of failing to detect infection (y-axis) given a certain prevalence (colour), sample size (x-axis) and test sensitivity (facets).

Table 2 provides an overview of the required sample at a certain 'confidence level' given the source prevalence, test sensitivity and batch size. For example, if we assume that an acceptable risk of undetected disease is 0.5%, or in other words the confidence level is 99.5%, we need to test 549 specimens (green cell in the table) from a batch size of 1000, given a prevalence of 1% and a sensitivity of the '*EURL PCR*' test (74.8%).

Table 2: Required sample size at different confidence levels, given the prevalence, sensitivity (Se) and batch size. A sensitivity (Se) of 0.748 represents the EURL PCR test, 0.95 is for a hypothetical test.

			Sample size required for confidence level:				
Prevalence	**Test Se**	**Batch size**	**99.9%**	**99.5%**	**99%**	**98%**	**95%**
0.01	0.748	1000	666	549	492	432	345
0.01	0.748	10,000	889	688	600	511	393
0.01	0.748	100,000	917	704	612	520	399
0.01	0.95	1000	523	431	387	339	271
0.01	0.95	10,000	699	541	472	402	309
0.01	0.95	100,000	721	554	481	409	313
0.02	0.748	1000	388	309	273	236	185
0.02	0.748	10,000	451	347	302	257	197
0.02	0.748	100,000	458	351	305	259	199
0.02	0.95	1000	305	243	215	185	145
0.02	0.95	10,000	354	273	237	202	155
0.02	0.95	100,000	359	276	240	204	156
0.04	0.748	1000	209	164	143	123	95
0.04	0.748	10,000	226	173	151	128	98

0.04	0.748	100,000	227	174	152	129	99
0.04	0.95	1000	164	128	112	96	75
0.04	0.95	10,000	177	136	118	100	77
0.04	0.95	100,000	178	137	119	101	77

Even if we consider a confidence level of 99% acceptable, we fail to detect presence of disease in 1% of the cases. If we repeat testing and releasing the negative tested batches several (x) times, we end up with a probability of an introduction that grows according to the following relationship:
$1 - (1 - 0.01)^x$.

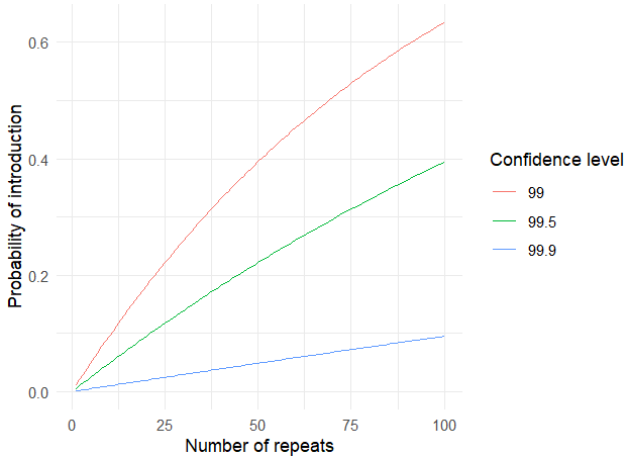

Figure 2. The probability of introduction of disease after an (x) number of repeated releases where a confidence level of 99%, 99.5% or 99.9% is used.

3.2 Re-testing

Comparison of testing a batch once with a certain sample size or testing and retesting with half the sample size did not result in different required sample size or probabilities of detection given a fixed sample size.

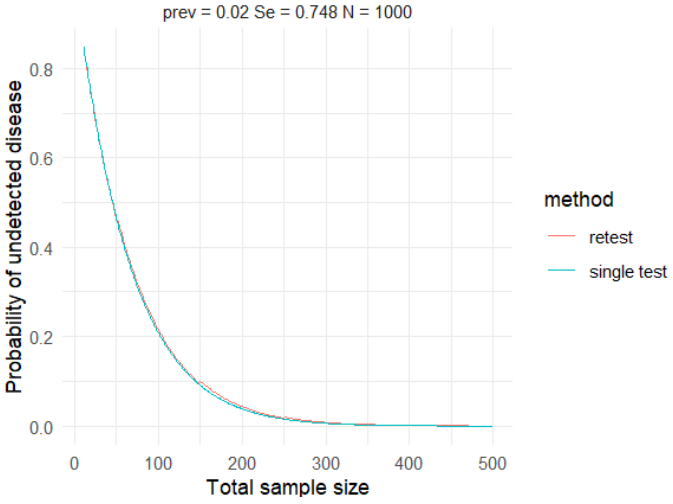

Figure 3. Single test versus retesting shows overlapping lines, here shown for the scenario of source prevalence of 2%, sensitivity of 74.8% and batch size of 1000. The total sample size is the sum of the two samples in the retest scenario.

4 Conclusions/Recommendations

- Assumptions on the source-prevalence and the required 'confidence level' influence the sample size required to assess freedom of disease.
- With repeated releases of batches the risk of introduction increases, also when testing for freedom of disease with a high confidence level.
- Repeated testing under the assumption that no changes in infection status, or diagnostic performance occur lead to no benefit (or harm).
- When taking into account the required amount of tissue for the test, we assume that there is no change in sensitivity of the test. Thus individuals of larvae or small spat (<6 mm) can be merged together to the required amount of tissue and subsequently be regarded as 1 unit in the calculations above.
- In order to reduce the number of tests, and thus costs, oysters can be pooled into a single sample (e.g. 5 oysters per samples). However, this will affect the test sensitivity.
- Note: Understanding whether the introduction of infected oysters would result into ongoing circulation (establishment) of *B. ostreae* in the target area was out of the scope of this assessment.

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