

Bonamia infection in native oysters (*Ostrea edulis*) in relation to European restoration projects

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Abstract:

1. There is a growing effort throughout Europe to restore populations of native oysters (*Ostrea edulis*), with the ecological objective of enhancing ecosystem biodiversity and resilience.
2. The introduced parasite, *Bonamia ostreae*, caused catastrophic mortalities during the 1980s, furthering the decline of this species, and is now present throughout much of the natural range of *O. edulis*. It is therefore important that restoration attempts avoid further introduction and spread of this parasite, which can cause lethal infections of *O. edulis*.
3. This article presents a comprehensive overview of the scale and distribution of current infection, transmission pathways and preventive measure guidelines, focusing on the seas, inlets and estuaries of North-West Europe, where most ecological restoration attempts for the native European oyster have occurred so far.
4. This is critical information for restoration project planning in which the risk of *Bonamia* infection must be taken into account.

Keywords: Coastal, Disease, Invertebrates, Restoration, Subtidal

1. Introduction

44 The European native (or 'flat') oyster (*Ostrea edulis*) was once abundant throughout many coastal
45 European waters and offshore areas of the North Sea (Figure 1), where it was found in dense
46 aggregations (Möbius, 1877). However, *O. edulis* suffered substantial declines throughout the 19th and
47 20th centuries. It is now extirpated from much of its range (Beck *et al.*, 2011) and is listed as a
48 threatened and declining habitat by OSPAR (OSPAR Commission 2009). There is now a growing effort
49 throughout Europe to restore populations of this habitat building species, with the aim of enhancing
50 biodiversity and ecosystem resilience (Pogoda *et al.*, 2017).
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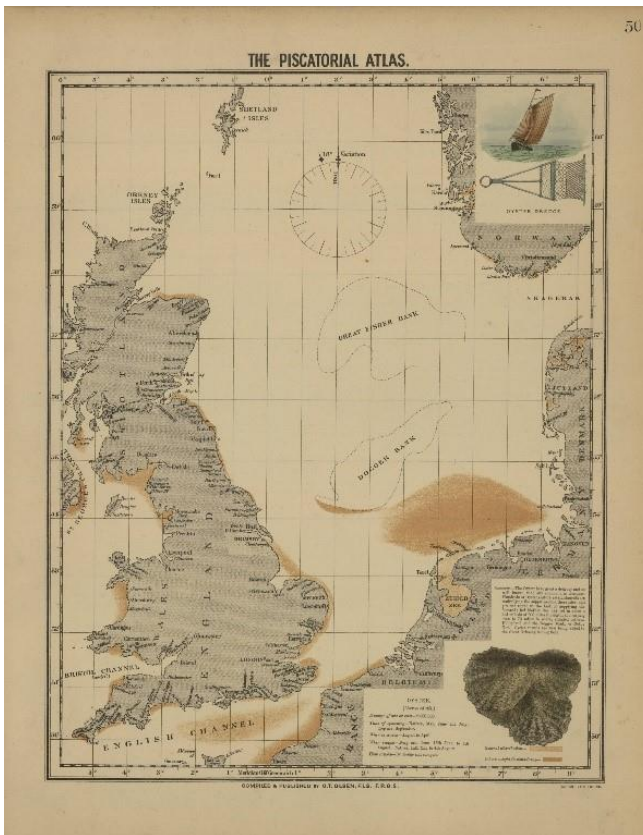


Figure 1: 19th century occurrence of *Ostrea edulis* in Olsen's *Piscatorial Atlas of the North Sea* (Olsen, 1883).

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53 While the initial collapse of *O. edulis* populations was largely driven by overfishing (Houziaux *et al.*,
54 2008; Gercken & Schmidt, 2014; Pogoda, 2019), the emergence of parasites such as *Bonamia ostreae*
55 and *Marteilia refringens* during the 20th century resulted in substantial mortalities, furthering a
56 renewed widespread decline of *O. edulis* (Laing *et al.*, 2006), in particular in aquaculture of this species
57 along European coasts. These parasites are still present in several European ecoregions, with varying
58 virulence and impact. *Bonamia ostreae* is especially widespread in the seas and inlets of North-West
59 Europe, posing a threat to the success of oyster restoration projects. Biosecurity relating to *B. ostreae*
60 transmission and spread is therefore an essential consideration when planning and implementing
61 restoration of *O. edulis*.

62
63 Bonamiosis is an oyster disease which is generally caused by parasites of the genus *Bonamia*. *Bonamia*
64 infects immune system cells (haemocytes) of the genus *Ostrea*. *B. ostreae* is the parasite which causes
65 the severest *O. edulis* disease in European waters, hence it is the main subject of this article. It has
66 been the focus of substantial research within aquaculture settings (e.g. Grizel, 2013; Bougrier *et al.*,

67 1986; Arzul *et al.*, 2009; Arzul *et al.*, 2011), but the specific impact of the disease on attempts to restore
68 high densities of *O. edulis* on the seafloor and the appropriate management to use in this setting
69 remains a knowledge gap. Current oyster restoration projects in Europe are seeking to increase the
70 density and extent of *O. edulis* to levels at which the species can be considered a self-sustaining
71 population. Since parasite prevalence probably increases with density (Engelsma, 2010), the risk of
72 disease incidence may increase through restoration attempts. This should obviously be avoided.

73
74 Because of this, it is important that restoration efforts comprehensively consider the risk posed by *B.*
75 *ostreae* and avoid its further spreading (Pogoda *et al.*, 2019). This is strongly encouraged by NORA,
76 the Native Oyster Restoration Alliance (for Europe). In order to avoid the risk of spreading *B. ostreae*
77 in restoration activities, it is important to consult the best available and most up to date knowledge on
78 *B. ostreae*. The current review presents a comprehensive overview of the current *B. ostreae* infection
79 distribution in North-West Europe, transmission pathways and preventive measures against the
80 disease, leading to recommendations for restoration project practices.

81
82 Many restoration projects in North-West Europe are currently being undertaken, as shown in Figure
83 2. There are numerous other sites where *O. edulis* are managed for aquaculture and food production,
84 but for Figure 2 only *O. edulis* restoration projects which are being undertaken to improve biodiversity
85 and habitat quality are selected.

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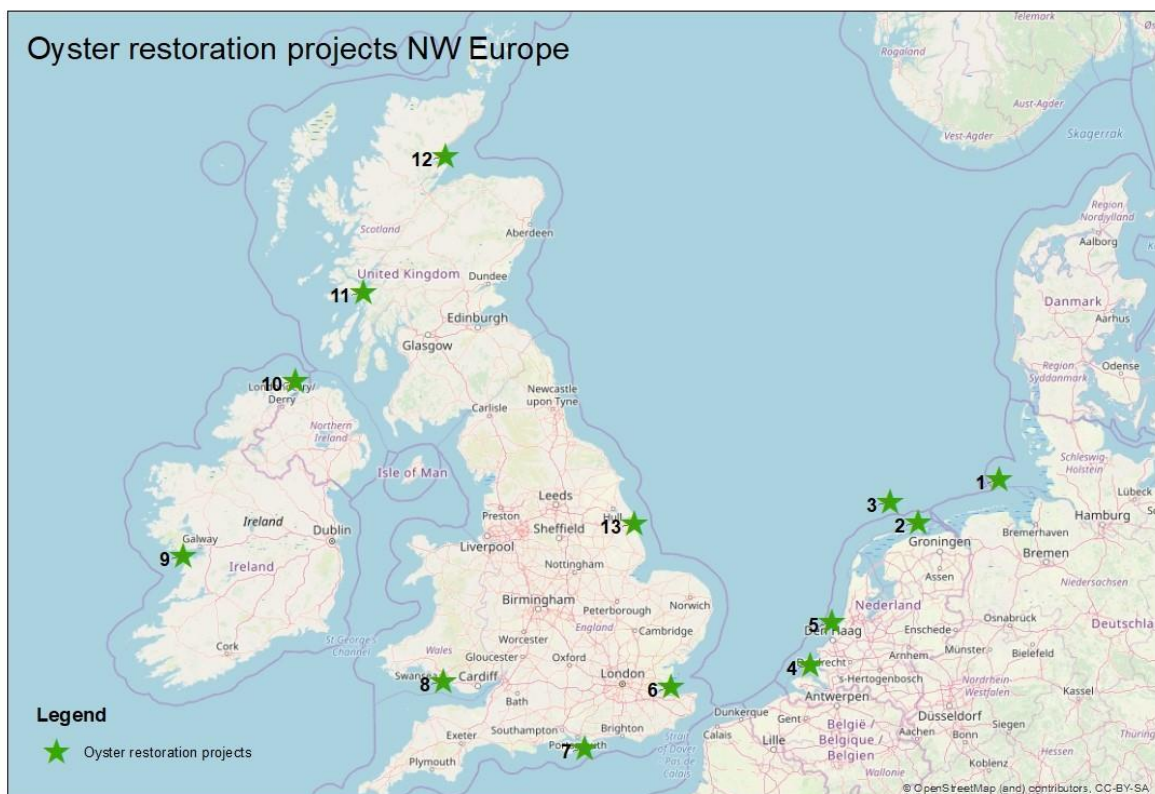


Figure 2: Impression of current *O. edulis* restoration attempts in North West Europe. Green asterix denotes restoration project. See table 1 in the Annex for the corresponding information on depicted restoration projects.

87

88

89 **2. Methods**

90 The urgent need to summarize the existing information regarding the *Bonamia* infection, its potential
91 impacts and management strategies for *O. edulis* restoration in Europe was recognised within the
92 NORA community with the initiation of the first European *O. edulis* restoration projects. An initial
93 review of the existing scientific, peer reviewed literature on the disease was presented at the 1st
94 NORA conference, of November 1-3, 2017 in Berlin. The article was extended and refined on the
95 basis of discussions during the conference and a second draft was presented and discussed at the 2nd
96 NORA Conference of May 21-23, 2019 in Edinburgh. In addition, experts on specific topics were
97 involved, resulting in the current author collective.

98

99 The basic data on geographical distribution of the *Bonamia* infection was obtained through a survey
100 of the relevant literature and public animal disease databases, such as (WAHIS, 2020). There is a
101 delay time between detection of the disease and publication in these sources, so that the NORA
102 community was consulted to obtain the most up-to-date information (until January 2020). The result
103 is presented in par. 4.2.

104

105 Since various terms are adopted in the literature to indicate the disease status, potentially leading to
106 confusion, the terminology in this article is here defined as:

- 107 • Oysters which are demonstrated to be infected are referred to as '***Bonamia*-infected**'
- 108 • Oysters originating from a region where *Bonamia ostreae* is present, are referred to as
109 '***Bonamia*-exposed**'.
- 110 • Oysters originating from a (also historically) *Bonamia*-free region, or demonstrated to be free of
111 the infection by adequate testing, are called '***Bonamia*-free**'.
- 112 • In the theoretical case that an oyster without infection is produced from a *Bonamia*-exposed
113 population, these are called '***Bonamia*-negative**'.

114

115 The first three terms are also adopted to indicate the infection status of oyster growing areas.

116

117 **3. What is bonamiosis and which species does it affect?**

118 Bonamiosis is a disease caused by unicellular parasites of the genus *Bonamia* (Culloty & Mulcahy, 2007;
119 Arzul & Carnegie 2015), included in the protozoan group Haplosporida, within Ascetosporea (Bass *et*
120 *al.*, 2019). Three *Bonamia* species have been characterized: *Bonamia ostreae* (Pichot *et al.*, 1980), *B.*
121 *exitiosa* (Hine *et al.*, 2001; Berthe & Hine, 2003) and *B. perspora* (Carnegie *et al.*, 2006). The parasite
122 named *B. roughleyi* (Farley *et al.*, 1988) was erroneously attributed to the genus *Bonamia* (Carnegie *et*
123 *al.*, 2014).

124 The host range of *B. ostreae* and *B. exitiosa* includes multiple species of the genus *Ostrea*. Besides *O.*
125 *edulis*, oyster species that are documented to be infected with *Bonamia* spp. are of the genera
126 *Crassostrea*, *Saccostrea* and *Dendostrea*, but with less severe consequences for the remaining
127 populations (Laramore *et al.*, 2017). See par. 5.3 for further discussion.

128

129 In Europe, *Bonamia exitiosa* infects *O. edulis* in Galicia (Abollo *et al.*, 2008; Ramilo *et al.*, 2014), and it
130 has been detected in Catalonia (Carrasco *et al.*, 2012), Italy (Narcisi *et al.*, 2010), France, UK (Longshaw
131 *et al.*, 2013) and Portugal (Batista *et al.*, 2016). *Bonamia perspora* is considered of less relevance in
132 Europe, since it has yet only been reported in *O. stentina* in North Carolina, USA (Carnegie *et al.*, 2006).

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Several other oyster diseases such as marteiliosis, due to *Marteilia refringens*, should also be considered within the framework of oyster restoration projects, but this article focuses on *B. Ostreae*, since this is currently considered to pose the most serious disease threat to *O. edulis* in North-West Europe.

4. *Bonamia ostreae* in North-West Europe

4.1 Introduction of *Bonamia ostreae* in Europe and its consequences

In Europe, oyster production and fishing activity was extensive during the 19th century. After severe declines in oyster stocks, related to unsustainable fishing pressure, large scale oyster translocations were undertaken in order to revive depleted populations. At the beginning of the 20th century the industry started to suffer from its first disease driven mortalities.

The oldest epizooty affecting flat oysters and related in the literature took place in France, The Netherlands and UK from 1920 to 1927 (Grizel, 1985; Héral, 1990). Although no infectious organism had really been incriminated (Orton, 1924 a; Orton, 1924b) described several abnormal cellular figures looking like intracellular parasites. During this period, the production drastically declined. The disease was retrospectively identified as probably caused by the flagellate protozoan *Hexamita* and associated with high laying densities as found in the managed beds (Tubbs, 1999).

In 1930s and 1940s, shell oyster disease, caused by the fungus *Ostracoblabe implexa* (Alderman & Jones, 1970; Alderman, 1985), caused severe losses to the Dutch oyster industry and, to a lesser extent, the French industry. This disease was overcome by changing some common practices in the culture procedures (Korringa, 1951; Korringa, 1976).

In 1968 in Aber Wrac’h, an inlet on the North-West coast of Brittany (France), the parasite *M. refringens* was diagnosed in oysters (Grizel *et al.*, 1974; Culloty & Mulcahy, 2007), causing large-scale mortalities in *O. edulis*. In 1979, a second parasite – *B. ostreae* – was discovered in L’Ile Tudy, at the south-west coast of Brittany (Pichot *et al.*, 1980), probably originating from the coastal waters of California (Elston *et al.*, 1987). This infection caused additional large-scale mortality and spread rapidly following its introduction, primarily due to the movements of infected oysters to new grow-out areas, or by careless movements of infected oysters with other shellfish (Culloty & Mulcahy 2007).

In France, a 93% reduction in yield was recorded between early 1970 and 1982 due to bonamiosis (Laing *et al.*, 2006). Overall, European production of *O. edulis* fell from 29.595 tons in 1961 to 5921 tons in 2000 (Culloty & Mulcahy, 2007). The impact of the diseases caused by *M. refringens* and *B. ostreae* resulted in a shift to the rearing of *Crassostrea gigas* and *O. edulis* production to remain low throughout the 1990s and beginning of the 21st century (Culloty & Mulcahy, 2007; Haenen *et al.*, 2011).

Being a serious oyster disease, Bonamiosis is notifiable to the World Organization for Animal Health (OIE, 2019) and it is included in the list of non-exotic diseases entailed in the EU Council Directive regulating aquatic animal health issues (EU, 2006). Movement of oysters from infected areas to infection-free areas poses an unacceptable biosecurity risk, yet the limited sources of *Bonamia*-free *O. edulis* spat or adults from historically *Bonamia*-free areas to be used as restoration broodstock pose a

178 challenge to restoration efforts. Understanding the historical spread, present infection status and
179 current knowledge of immunological responses to this infection is imperative for sustainable
180 restoration efforts.

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182 **4.2 Current spread of *Bonamia Ostreae* in North-west Europe**

183 The majority of *O. edulis* populations in Europe are now infected by *B. ostreae* (Figure 3). The data base
184 underlying Figure 3, with location names, source and years of first recorded *B. ostreae* presence (if
185 available) is presented in Annex Table 2. The ultimate data underlying the map and the table are from
186 January 2020.

187

188 *Bonamia ostreae* is thought to have first spread through oyster cultures in France (Elston *et al.*, 1987)
189 and Spain (Cigarria *et al.*, 1997) before reaching other European coastal waters within a decade (Culloty
190 & Mulcahy, 2007). Bonamiosis reached the United Kingdom in 1982 and Ireland in 1987 (Culloty &
191 Mulcahy, 2007). Some bays and inlets in the UK and Ireland, however, have thus far remained
192 *Bonamia*-free (Laing *et al.*, 2015).

193

194 In Norway, oyster cultures have been regularly surveyed since 2008 and an infection detection was
195 reported for 2009 in the Langestrand area (OIE, 2009). However, the parasite has not been detected
196 since at this location during examinations carried out by the National Veterinary Institute (Mortensen
197 *et al.*, 2016; Mortensen *et al.*, 2018). Hence, the status reported in Figure 3 is ‘uncertain’ for the
198 Langestrand location. Repeated surveys at other Norwegian locations showed no *Bonamia*-infection
199 (Mortensen *et al.*, 2018), so these are reported as *Bonamia*-free in Figure 3.

200

201 In Denmark, the main oyster culture area is Limfjord, which remained *Bonamia*-free for a long time
202 (Møllgaard, 2008). *B. ostreae* was recently reported at very low prevalence in the Nissum Bredning,
203 in the western part of Limfjorden (ICES, 2018; Madsen, 2017), which means that Limfjord is now
204 considered a *Bonamia*-infected area, regardless of the fact that there has been no increased mortality
205 (Madsen, 2017).

206

207 The *Bonamia* status in Dutch waters is generally ‘infected’. Yet, a small *O. edulis* population was
208 recently discovered in the Dutch Wadden Sea, which was tested by performing DNA-analysis on a large
209 number of larvae produced in a hatchery. These were reported free from *Bonamia* (Jacobs *et al.*, to be
210 submitted). However, since no adult oysters were tested, the *Bonamia* status of the area has to be
211 considered as ‘uncertain’. Open-sea areas in Dutch waters marked as *Bonamia*-free in Figure 3
212 represent isolated restoration projects, for which *Bonamia*-free oysters (from Norway) have been
213 employed. In an early restoration (2017) restoration project off the west coast of The Netherlands
214 *Bonamia*-infected oysters were deployed. These oysters could not be retraced, but the location is
215 marked as ‘infected’ nonetheless.

216

217 In December 2019 it was discovered that the Lynn of Lorne, Loch Creran, Loch Etive and Dornoch Firth
218 oyster populations in Scotland are infected by *Bonamia* (Scottish Government, 2020).

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Figure 3: Occurrence of *B. ostreae* infection in North West Europe. The colour of the marked points indicate the infection status of the present oyster population as revealed by our survey.

Explanation of legend:

- When a location is marked as **infected**, this means that one or more oysters from this area have been tested *B. Ostrea* positive.
- When a location is marked as **not infected**, this means that no *B. Ostrea* has been detected with regular surveys and tests to date.
- When a location is marked as **uncertain**, this means sources on the *Bonamia* status are contradictory, not present or unknown.

For details on prevalence of *Bonamia* in marked locations, see table 2 in the Annex.

221 **5. Characteristics of *Bonamia ostreae* and bonamiosis and the relevance for** 222 **restoration practice**

223

224 **5.1 Infection and disease development in oysters**

225 Once present, the *Bonamia* parasite spreads rapidly through *O. edulis* beds (Culloty *et al.*, 1999).
226 Although pathways of infection are not fully known, *O. edulis* is susceptible to infection by *B. ostreae*
227 at all life-history stages, including during larval phases (Lynch *et al.*, 2005; Arzul *et al.*, 2011). Male and
228 female oysters are equally susceptible to infection (Culloty & Mulcahy, 1996). An initial 'latent' period
229 can mask the infection from detection for anything from four weeks to several months (Culloty *et al.*,
230 2001).

231

232 *Bonamia ostreae* is an intracellular parasite (2–5 µm) that infects the haemocytes and, occasionally,
233 branchial epithelium (ectoderm) of the oysters (Montes *et al.*, 1994; Arzul & Carnegie, 2015).
234 Haemocytes are suspended in the haemolymph fluid, which is a plasma similar to the blood in
235 vertebrates. One of the functions of haemocytes is to detect and destroy pathogens, but *O. edulis*
236 haemocytes fail to destroy *Bonamia*. There is evidence that the parasite inhibits or blocks molecular
237 weapons of oyster haemocytes to destroy pathogens (Hervio *et al.*, 1991; Gervais *et al.*, 2016; Gervais
238 *et al.*, 2018; 2 Gervais *et al.*, 019).

239

240 The infection usually develops through infiltration of infected haemocytes into the tissues of the gills
241 and mantle and around the gut. In its severe state it causes loss of the normal architecture of the gills,
242 the digestive gland, the gonad and other organs leading to general dysfunction and ultimately death
243 of the oyster (Culloty & Mulcahy, 2007). Bonamiosis usually causes highest mortality in oysters that
244 are 3 years or older, although younger infected oysters may also suffer mortality (Lynch *et al.*, 2005).
245 Sometimes, the effect of the disease is sublethal, reducing the host's ability to cope with additional
246 stressors such as changes in water temperature, translocation to other environments or reproductive
247 activity (Dijkema, 1990; van Banning, 1991) and increasing host susceptibility to other micro-
248 organisms. Eradication of, or treatment against, *B. ostreae* is not considered possible (Morga *et al.*,
249 2017).

250

251 The parasite occurs throughout the year, but prevalence of infection tends to be highest in spring and
252 summer, with the peak of prevalence at the end of winter to spring in most of the infected countries
253 in Europe (Grizel *et al.*, 1988; Culloty & Mulcahy, 1996; Engelsma *et al.*, 2010).

254

255 **5.2 Detection methods of infection by *B. ostreae* at individual and population level**

256 Detection of *Bonamia* presence in the source *O. edulis* population for restoration purposes is essential
257 to avoid accidental spreading of the infection. Testing should also be performed if an *O. edulis*
258 population is already present in the restoration area in order to determine any previous presence of
259 the parasite.

260

261 *Bonamia ostreae* infection is often difficult to detect visually in the oyster, but gross signs can
262 occasionally be observed including yellow discoloration in the gills, extensive lesions, including
263 perforated ulcers in the connective tissues of the gills, mantle and digestive gland. Standard diagnostic

264 methods use cytology (haemolymph smears or tissue imprints) and histopathology to screen oyster
265 tissues, after staining the sample (da Silva & Villalba, 2004) .

266

267 DNA-techniques, based on the Polymerase Chain Reaction (PCR), are now widely used, due to their
268 high specificity and ability to detect very low infection levels (Flannery *et al.*, 2014a). New species-
269 specific molecular methods are available (Ramilo *et al.*, 2013) and their use is recommended in
270 European regulation (EU, 2015). These species-specific tools (Ramilo *et al.*, 2013; Flannery *et al.*, 2014a;
271 Batista *et al.*, 2016) confer high sensitivity and can detect a lower degree of infection/presence than
272 histological analysis. Yet, it may also yield false positive detections. The lower sensitivity of more dated
273 primers that are currently recommended by the World Organization for Animal Health (OIE, 2019) may
274 provide underestimations of prevalence within a population (Helmer *et al.*, 2019).

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276 So, compared to histology, DNA-techniques appear to be more sensitive. However, they are indicative
277 of the presence of *B. ostreae* DNA and not of an infection: histology remains a key technique to confirm
278 an infection especially in a previously *Bonamia*-free population or region.

279

280 Even if the prevalence of the infection in a population is low, it is crucial to be able to detect it. An
281 important factor in determining whether a population can confidently be assessed for its *Bonamia*
282 infection status is sample size. The EU prescribes a minimum sample size of 150 individual oysters in
283 Annex I, part 5 of (EU, 2015). The document does not explain the requirements and assumptions
284 underlying this number, but by using basic statistics as provided by the World Organization for Animal
285 Health (OIE, 2008) these can be reconstructed as:

- 286 • required confidence level: 95%;
- 287 • *Bonamia* prevalence in the *O. edulis* population to be tested: 2%;
- 288 • sensitivity of the testing method: 95%.

289

290 More extensive recommendations for *Bonamia* survey and detection methods are given in (OIE, 2019).

291

292 **5.3 Spreading mechanisms of the *Bonamia* infection**

293 Transmission pathways of *B. ostreae* may occur directly from parent oysters to larvae, but also via the
294 water column, probably via filtration (Culloty & Mulcahy, 2007; Arzul *et al.*, 2011). The mechanism of
295 transmission is not fully understood, though some mechanisms and factors are described in (Engelsma
296 *et al.*, 2014).

297

298 The maximum transmission distance is also unknown. It could be relatively small, since infection
299 prevalence tends to increase with oyster population density (Engelsma *et al.*, 2010) and *Bonamia*- free
300 and -infected *O. edulis* areas are observed to exist at a close distance to each other, e.g. in bays and
301 inlets in South-west England (Figure 3). However, since the infection can be transferred through water
302 currents and also larvae (which remain in the water phase for 11-30 days) potentially large dispersal
303 distances (10 km or more) can occur, depending on the local hydrogeographic regime. The main
304 infection vector is, however, considered to be shellfish transfers of infected *O. edulis*. Hence, EU
305 regulation against the spreading of the infection focuses on quarantining infected areas where
306 transport of *O. edulis* from infected to non-infected areas is prohibited (EU, 2006).

307

308 Given that the infection can be transmitted through larvae and the water phase, once present on an
309 oyster bed, *B. ostreae* cannot be eradicated (van Banning, 1991). *O. edulis* are not the only shellfish
310 species to transmit *B. ostreae* (Engelsma *et al.*, 2014; Laramore *et al.*, 2017). Contrary to initial
311 evidence, which suggested that *C. gigas* was not susceptible to infection (Culloty *et al.*, 1999), it is now
312 believed that it may indeed act as either a paratenic or dead-end host for both *B. ostreae* and *B. exitiosa*
313 (Lynch *et al.*, 2010; Helmer *et al.*, 2019). This should be investigated further as infection and
314 transmission via this highly abundant and commercially produced species could have implications for
315 restoration of *O. edulis* and the transport of commercial stock could exacerbate the spread of *Bonamia*
316 species. Non-bivalve species may also serve as vectors, such as the brittle star *Ophiothrix fragilis* (Lynch
317 *et al.*, 2007).

318

319 **5.4 Sensitivity of the *Bonamia* parasite to climate change**

320 General effects of climate change include higher temperature and (through dissolution of CO₂) lower
321 pH (Huthnance *et al.*, 2016). To our knowledge, there are no specific studies on the impact on the
322 prevalence and/or the mortality caused by *Bonamia* infection to *O. edulis* populations under these
323 climate change scenarios. (Arzul *et al.*, 2009) have, however, studied the survival of purified *Bonamia*
324 parasites using seawater from three different sources with pH values of 8.06, 7.06 and 6.5 under
325 different temperature regimes. The results showed significantly lower survival at 25°C compared to
326 4°C and 15°C. Regarding pH, an experiment *ad hoc* was not performed because seawater with different
327 pH values also differed in chemical composition, but the results showed a better survival of purified *B.*
328 *ostreae* (60 to 80%) in the seawater with pH = 8.06 and in that with pH = 7.06 than in artificial seawater
329 (survival less than 40%) with pH = 6.5, regardless of temperature and incubation time. It is worth noting
330 that *B. ostreae* exhibited high survival under the full range of pH and temperature conditions tested.
331 Besides, the tested range in temperature and pH is far greater than the actual changes in the variables
332 predicted in (Huthnance *et al.*, 2016) for the end of this century, so it seems unlikely that the *Bonamia*
333 parasite will be strongly negatively impacted by climate change in the near to medium term. Yet,
334 specific research on the interactions between climate change effects and *Bonamia* is needed to test
335 this hypothesis.

336

337 **5.5 Evidence for tolerance or resistance in existing *O. edulis* populations**

338 Disease-tolerance and disease-resistance are two physiological defence strategies demonstrated by *O.*
339 *edulis* in response to infection by the parasite *B. ostreae*. Disease resistance is when the parasite is
340 able to infect the host, but it is unable to multiply, reproduce and therefore to proliferate within the
341 host tissues. Resistant individuals have also demonstrated the ability to reduce parasite burden
342 (Råberg *et al.*, 2007; Ayres & Schneider, 2008; Morga *et al.*, 2017). Disease tolerance is when the host's
343 fitness is not greatly affected by the presence of the parasite, regardless of its successful proliferation
344 in host tissues (Ayres & Schneider, 2008; Råberg *et al.*, 2008). This balance between parasite and
345 tolerant host can be interrupted by stress, as any environmental pressure such as a change in abiotic
346 conditions or food supply can lead to immune imbalance, resulting in host mortality (Mydlarz *et al.*,
347 2006).

348

349 Although marine invertebrates lack the ability to develop pathogen specific antibodies, *O. edulis* from
350 *Bonamia*-exposed populations have demonstrated more resistance or tolerance to the parasite than
351 oysters from *Bonamia*-free populations (Hervio *et al.*, 1995; Culloty *et al.*, 2001; 2004; da Silva *et al.*,
352 2005). (Morga *et al.*, 2017) demonstrated a degree of disease resistance in *Bonamia*-exposed oysters,

353 with inhibiting phagocytotic activity to reduce the spread of parasites to further tissue, while
354 inducing in haemocytes the expression of genes associated with apoptosis, thus hampering parasite
355 proliferation within haemocytes.

356

357 Various studies in different countries have shown that oysters living in areas long-term affected by
358 bonamiosis (more than 20 years) survive exposure to *B. ostreae* much better than oysters living in
359 areas only-recently affected by the disease or in non-affected areas, indicating development of natural
360 resistance or tolerance of oysters to infection by the parasite over time (Elston *et al.*, 1986; da Silva *et al.*,
361 *et al.*, 2005; Flannery *et al.*, 2014b).

362

363 Selective breeding for resistance or tolerance has taken place in Cork Harbour, Ireland (Lynch *et al.*,
364 2014a). This has taken the form of large-scale breeding trials in spatting ponds, using four to five year
365 old survivors of the disease. In laboratory and field-based trials comparing the susceptibility of the Cork
366 Harbour *O. edulis* with Irish and European populations, the former have performed well (Culloty *et al.*,
367 2001; Culloty *et al.*, 2004). Again, the mechanism through which this occurs is unknown. Additionally,
368 pilot programmes have been performed in France (Baud *et al.*, 1997; Naciri-Graven *et al.*, 1998; Naciri-
369 Graven *et al.*, 1999) and Spain (da Silva *et al.*, 2005,) also showing that selective breeding leads to
370 significant increase of tolerance or resistance and survival.

371

372 (Culloty *et al.*, 2004) compared performance of oysters that had been selectively bred for resistance
373 to *B. ostreae* (Rossmore, Cork harbour, Ireland), and oysters from two areas where *Bonamia* has been
374 present for a long time (Lake Grevelingen, the Netherlands and Brittany, France) with oysters from
375 four *Bonamia*-free populations (Lough Foyle, Ireland; Tralee, Ireland; Lough Kishorn, Scotland; Mull,
376 Scotland). Oysters from all these locations were translocated to Cork Harbour (Ireland), Lake
377 Grevelingen (the Netherlands) and Brittany (France). The field trials indicated that Rossmore and Lake
378 Grevelingen oysters showed lower mortality compared to other populations. (Culloty *et al.*, 2004)
379 conclude that previous exposure in these populations has conferred some reduced susceptibility to
380 the parasite compared to *Bonamia*-free populations. In a follow-up study spat was produced in the
381 hatchery of Roem van Yerseke with broodstock from long-term exposed populations in Lake
382 Grevelingen and the Oosterschelde and a *Bonamia*-free population in Limfjord in Denmark. Spat of all
383 three groups was reared for 1 year in Lake Grevelingen. Survival was best in spat from Lake Grevelingen
384 (OYSTERECOVER, 2013). It was concluded that Grevelingen should be considered as a candidate stock
385 for starting a breeding programme in the Netherlands. Although this stock had the highest overall
386 prevalence of infection, it also had the greatest growth and survival rate indicating that it may have
387 formed some local tolerance to the disease. Appropriate design to avoid undesirable side-effects of
388 inbreeding or substantial reduction of the genetic variability of the species should be considered when
389 selecting oysters for resistance or tolerance.

390

391 The development of *B. ostreae* resistance and/or tolerance is a hopeful sign. Efforts to understand how
392 oysters become resistant against *B. ostreae* have increased in the last years; studying gene expression
393 associated with *B. ostreae* infection (Morga *et al.*, 2011; Morga *et al.*, 2017; Gervais *et al.*, 2016; Gervais
394 *et al.*, 2018; Gervais *et al.*, 2019) and comparing it between *O. edulis* stocks with different susceptibility
395 to the parasite (Pardo *et al.*, 2016; Morga *et al.*, 2017) are providing clues. Decreasing phagocytic
396 activity and increasing apoptosis (i.e. cell suicide) of haemocytes seem to be associated with increased

397 oyster resistance (Morga *et al.*, 2017; Gervais *et al.*, 2016; Gervais *et al.*, 2019), likely by restraining
398 parasite multiplication within haemocytes.

399
400 Genetic analysis has so far identified multiple genes indicating bonamiosis immunity, including OelAP
401 and OeFas-ligand gene expression, highlighting differences in wild-type and selectively bred oysters in
402 their ability to regulate apoptosis (Morga *et al.*, 2017). Comparison of gene expression profiles in
403 *Bonamia*-free and -infected oysters are producing suites of candidate resistance conferring genes (e.g.
404 Ronza *et al.*, 2018; Vera *et al.*, 2019) for testing and screening resistance (also see par. 5.2). Proteomic
405 approaches can also contribute to identify molecular markers of resistance to bonamiosis (de la Ballina
406 *et al.*, 2018)

407
408 Given the importance of promoting resistance and/or tolerance on the one hand, and the absolute
409 need to avoid the spread of *Bonamia* on the other, this is a critical, though challenging, area of
410 research.

411

412 **5.6 Biosecurity measures**

413 As the transfer of stocks of *O. edulis* is considered to be responsible for the introduction of bonamiosis
414 in Europe (Bromley *et al.*, 2016) , biosecurity measures rely on the prohibition of transfer of live or
415 dead oysters, of any age class, from an infected area. This is mandatory under current EU regulations
416 (EU, 2006). In accordance to this regulation, all oyster transports are subject to licensing, according to
417 EU and/or national regulation. The project organizer should therefore always apply for a transport
418 licence (and other relevant licences) from the competent authorities in the country where the
419 restoration project is undertaken and adhere to licence conditions at all time.

420
421 Upon transfer of oysters to sensitive locations, such as the restoration project area, hatcheries, etc.,
422 measures have to be put in place to limit spreading of the disease as much as possible. These should
423 include the quarantine of oysters, combined with analysis for the detection of *B. ostreae* on a sample
424 of the oysters, applying the techniques explained in par. 5.2. Most techniques lead to the destruction
425 of the sample, but a non-destructive method (analysing samples of tissue collected from previously
426 anaesthetized oysters (Kamermans et al, submitted)) is being developed.

427

428 **5.7 Production of oysters which are simultaneously *Bonamia*-free and *Bonamia*- 429 tolerant/resistant**

430 Production of oysters which are simultaneously *Bonamia*-free and *Bonamia*-tolerant should be
431 technically feasible. Infection of a population by *Bonamia* does not result in the total eradication of
432 that population. Within the remaining population there will always be uninfected as well as infected
433 individuals. Following long term exposure to the parasite, these uninfected individuals can be
434 identified within the population, and spat derived from them in a hatchery can be non-infected.
435 *Bonamia*-infection in this new generation can be reliably detected with PCR/DNA analysis, given the
436 correct minimum amount of spat tested. Hence, a *Bonamia*-free broodstock can be established in a
437 hatchery and, if managed properly (with quarantine measures), non-infected spat ready to be relayed
438 can be produced from these. These oysters may have developed tolerance or resistance to the disease
439 (Kamermans at al., submitted).

440

441 This is potentially very useful for restoration projects, since international regulations and national
442 policies aim to prevent the transfer of diseases to new areas, but protection against disease is desired,
443 in case it does appear in a newly established bed. Recently, the first step in this process has been taken.
444 A novel, non-destructive screening method to determine the status of the oyster with regard to
445 *Bonamia* was developed and the selected *Bonamia*-free broodstock produced *Bonamia*-free spat
446 (Kamermans *et al.*, submitted). Further analysis into the genetic profile of these spat is underway to
447 identify any genes that can be used as markers for resistance.

448

449 **5.8 Maintaining genetic diversity**

450 Genetic differentiation exists between Atlantic, Mediterranean and Black Sea native oyster
451 populations (Sobolewska & Beaumont, 2005; Launey *et al.*, 2002; Diaz-Almela *et al.*, 2004). Native
452 oysters have been cultivated since Roman times, and translocations, especially during the 1800s,
453 were most intense between various North East Atlantic populations, with translocations taking place
454 to a lesser extent between North East Atlantic and Mediterranean populations (Bromley *et al.*, 2016).
455 This can explain the moderate genetic differentiation between Atlantic and Mediterranean *O. edulis*
456 populations and a tendency for Atlantic populations to be even less differentiated than
457 Mediterranean ones (Launey *et al.*, 2002). However, (Vera *et al.*, 2016) studied oyster populations in
458 the Netherlands, Denmark, Ireland, England, France and Spain with detailed methods and revealed
459 systematic genetic differences between native oysters in three geographical regions: (1) The
460 Netherlands and Denmark; (2) France, Ireland and England; and (3) Spain. In addition (Guitierrez *et*
461 *al.*, 2017) showed high genetic similarity in *O. edulis* between Norway, Lake Grevelingen and Maine.

462 The selection of resistant oysters involves reproduction with *Bonamia*-free broodstock in a hatchery.
463 Spat produced in a hatchery has a lower genetic diversity than pond production or spat collection in
464 the field (Lallias *et al.*, 2010). Thus, it is important to maintain genetic diversity in hatchery
465 production through regular replacement of broodstock oysters, with new individuals from outside
466 waters (Ryman & Laikre, 1991).

467

468 **6. Recommendations for restoration practice**

469

470 **6.1 Avoidance of spreading diseases in general**

471 Since *O. edulis* have been extirpated from much of their natural range, restoration often involves
472 introduction of a breeding population. Care should be taken that this introduction does not lead to
473 spreading of diseases, impacting shellfish or other species. This article focuses on the *Bonamia*-
474 infection, since this is considered to be the most severe native oyster disease in North-West Europe,
475 but it should be investigated whether other diseases, such as *Marteilia refringens*, are present in the
476 breeding population and whether these can have a negative impact in the project area. If so, the type
477 of measures recommended in this article to avoid spreading of the *Bonamia* infection should be
478 applied to these other diseases.

479

480 **6.2 Detection of *Bonamia* presence and adherence to licence procedures**

481 The recommendations in the following paragraph give guidance to using *Bonamia*-exposed or
482 *Bonamia*-free oysters in the relevant circumstances. It should be noted that even when these
483 recommendations are adhered to, all oyster transports are subject to licensing, according to EU
484 and/or national regulation. The project organizer should therefore always determine the *Bonamia*-

485 infection status of the breeding population, applying the detection methods and following the EU-
486 regulation explained in par. 5.2 and par. 5.6. In addition, a transport licence (and other relevant
487 licences) should be applied for at the competent authorities in the country where the restoration
488 project is undertaken and licence conditions should be adhered to at all time. While undergoing the
489 detection process, the oysters to be transported or introduced should be kept in quarantine.

490 **6.3 Should *Bonamia*-exposed or *Bonamia*-free oysters be used for restoration purposes?**

491 A pertinent question is whether to operate with *Bonamia*-exposed or *Bonamia*-free oysters. In 2017
492 NORA (Native Oyster Restoration Alliance) members drafted and agreed upon the following set of
493 guidelines when employing *O. edulis* restoration projects (Pogoda *et al.*, 2017; Pogoda *et al.*, 2019).

494

495 **1. If an oyster (*O. edulis* or otherwise) population is already present in the restoration area and the** 496 **population is *Bonamia*-free:**

497 Only *Bonamia*-free oysters can be introduced even if close to a *Bonamia*-infected region or (sub)area.
498 As Figure 3 shows, there are several situations where a *Bonamia*-free area exists close to a *Bonamia*-
499 infected area, and spread of the infection must be avoided by restoration attempts.

500

501 **2. If an oyster (*O. edulis* or otherwise) population is already present in the restoration area and the** 502 **population is *Bonamia*-exposed:**

503 Either *Bonamia*-free or *Bonamia*-exposed oysters can be employed, but from a restoration
504 perspective it is recommended to introduce *Bonamia*-exposed oysters in these areas, since they may
505 have developed a certain level of tolerance or even resistance.

506

507 **3. If an oyster (*O. edulis* or otherwise) population is absent in the restoration area:**

508 Many current or planned restoration projects aim at reintroducing oyster populations in areas where
509 oysters themselves are not present anymore, such as the open North Sea, Channel or Irish Sea.

510

511 Arguments in favour of using *Bonamia*-free oysters in these open sea areas are:

- 512 ● It is guaranteed that the infection does not spread through the restoration attempt.
- 513 ● The oysters may be in a better condition, since they do not suffer from the illness, and therefore
514 may better survive displacement stress (see par. 5.1).

515

516 The argument in favour of using *Bonamia*-exposed oysters in the open sea is that the infection is
517 broadly present around these seas and eventually, the infection may reach the restoration area
518 sometime in the future, not only through *O. edulis*, but also through other hosts, possibly even
519 *Crassostrea gigas*. In that case *Bonamia*-exposed oysters, which may have developed tolerance or
520 resistance, could have an advantage.

521

522 A rational decision to use either *Bonamia*-exposed or *Bonamia*-free oysters is therefore subject to an
523 assessment of the risks involved (risk of infection, risk of high mortality due to displacement stress
524 combined with the infection etc.). However, it is impossible to make a reliable risk assessment on the
525 basis of current scientific knowledge so that application of the precautionary principle, i.e. by only
526 introducing *Bonamia*-free oysters in areas where no oyster population previously existed, is strongly
527 recommended. This recommendation holds for the whole open North Sea, Channel, Irish Sea and other
528 open sea areas.

529 It should be noted that there is ongoing research into production of *Bonamia*-free oysters, produced
530 from an infected, and therefore possibly *Bonamia*-tolerant or *Bonamia*-resistant population
531 (Kamermans *et al.*, submitted). Should the rearing of tolerant/resistant and yet *Bonamia*-free oysters
532 become possible, then this represents an opportunity to reduce the risk both of introducing the disease
533 to new areas and of suffering high mortalities should the disease appear at a later stage. In any case,
534 it should be absolutely guaranteed that these oysters are free from the infection before they can be
535 deployed. How this guarantee can be realized (detection accuracy, quarantine measures etc.) should
536 be researched and tested in detail and agreed by key experts before application in practice can be
537 considered.

538

539 **6.3. Recommendations for future research**

540 There are still many unknowns regarding the impact of *Bonamia* on *O. edulis* restoration activities,
541 such as the impact of oyster density, temperature and food availability on disease prevalence in
542 natural systems (zu Ermgassen *et al.* this issue). The importance of developing research to
543 understand both the mechanisms *Bonamia* tolerance or resistance, and ways in which scaling up the
544 production of tolerant or resistant spat for restoration purposes was also identified, and remains a
545 pressing issue.

546

547 For the time being it is important to emphasise that current best practice, from a legal as well as
548 nature conservation perspective, is to use *Bonamia*-free *O. edulis* for restoration efforts in situations
549 where no living oysters are currently present.

550

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