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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

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

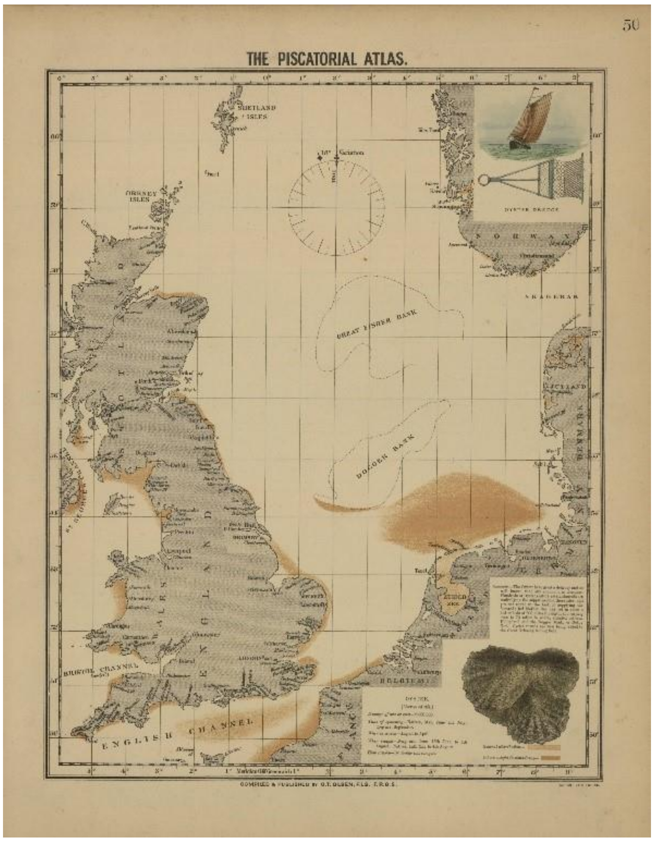

Figure 1: 19th century occurence of Ostrea edulis in Olsen's Piscatorial Atlas of the North Sea (Olsen, 1883).

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

Bonamiosis is an oyster disease which is generally caused by parasites of the genus *Bonamia*. *Bonamia* infects immune system cells (haemocytes) of the genus *Ostrea. B. ostreae* is the parasite which causes the severest *O. edulis* disease in European waters, hence it is the main subject of this article. It has been the focus of substantial research within aquaculture settings (e.g. Grizel, 2013; Bougrier *et al.,* 1986; Arzul *et al.,* 2009; Arzul *et al.,* 2011), but the specific impact of the disease on attempts to restore high densities of *O. edulis* on the seafloor and the appropriate management to use in this setting remains a knowledge gap. Current oyster restoration projects in Europe are seeking to increase the density and extent of *O. edulis* to levels at which the species can be considered a self-sustaining population. Since parasite prevalence probably increases with density (Engelsma, 2010), the risk of disease incidence may increase through restoration attempts. This should obviously be avoided.

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

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

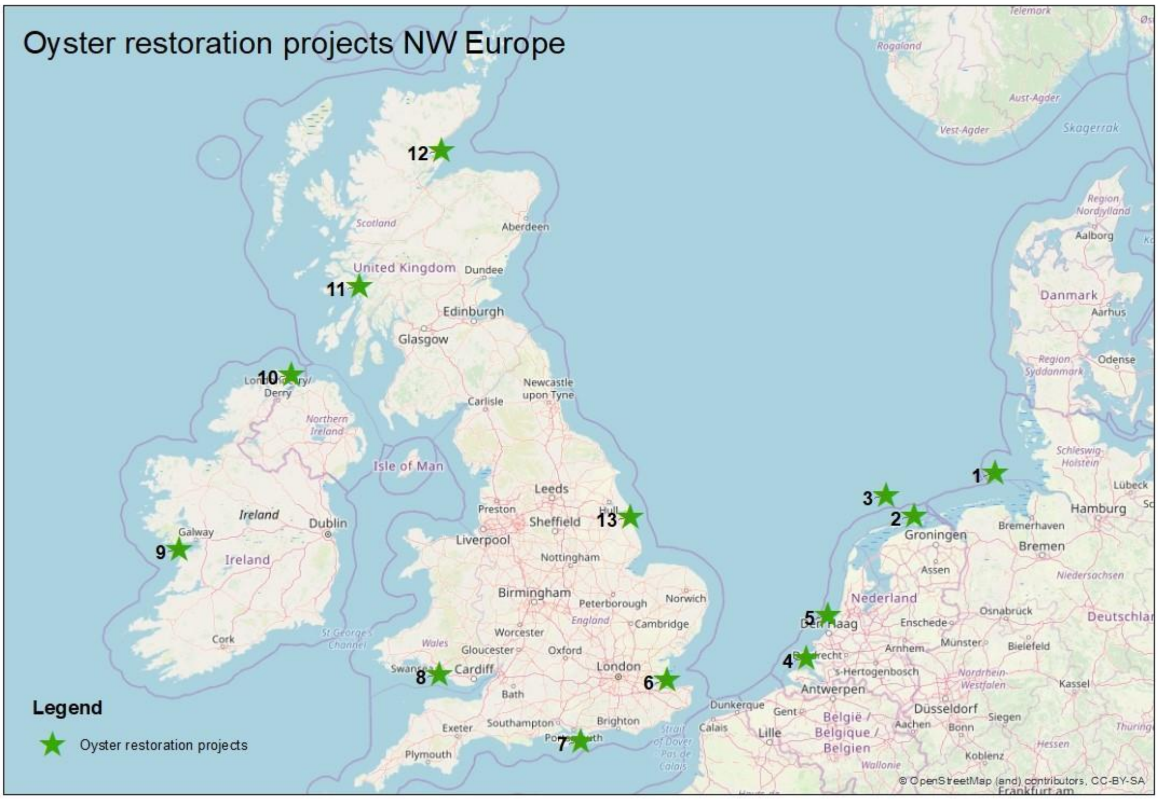

Figure 2: Impression of current O. edulis restoration attempts in North West Europe. Green asterix denotes restoration project. See table 1 in the Annex for the corresponding information on depicted restoration projects.

2. Methods

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

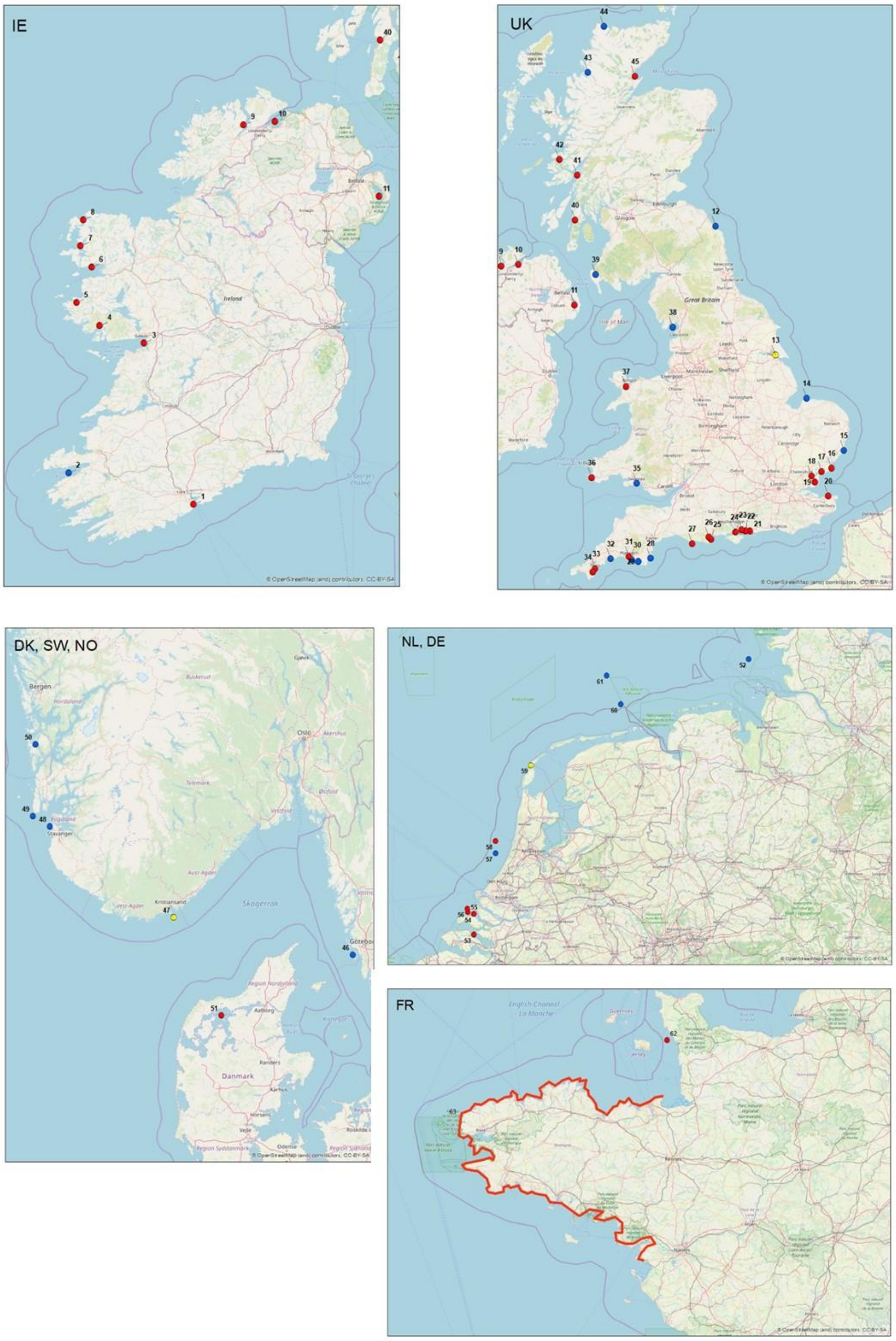

Figure 3: Occurrence of B. ostreae infection in North West Europe. The colour of the marked points indicate the infection status of the present oyster population as revealed by our survey.
Explanation of legend:

- *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.

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

5.1 Infection and disease development in oysters

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

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

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

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

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

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

Bonamia ostreae infection is often difficult to detect visually in the oyster, but gross signs can occasionally be observed including yellow discoloration in the gills, extensive lesions, including perforated ulcers in the connective tissues of the gills, mantle and digestive gland. Standard diagnostic methods use cytology (haemolymph smears or tissue imprints) and histopathology to screen oyster tissues, after staining the sample (da Silva & Villalba, 2004) .

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

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

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

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

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

5.3 Spreading mechanisms of the *Bonamia* infection

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

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

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

5.4 Sensitivity of the *Bonamia* parasite to climate change

General effects of climate change include higher temperature and (through dissolution of CO_2) lower pH (Huthnance *et al.,* 2016). To our knowledge, there are no specific studies on the impact on the prevalence and/or the mortality caused by *Bonamia* infection to *O. edulis* populations under these climate change scenarios. (Arzul *et al.,* 2009) have, however, studied the survival of purified *Bonamia* parasites using seawater from three different sources with pH values of 8.06, 7.06 and 6.5 under different temperature regimes. The results showed significantly lower survival at 25°C compared to 4°C and 15°C. Regarding pH, an experiment *ad hoc* was not performed because seawater with different pH values also differed in chemical composition, but the results showed a better survival of purified *B. ostreae* (60 to 80%) in the seawater with pH = 8.06 and in that with pH = 7.06 than in artificial seawater (survival less than 40%) with pH = 6.5, regardless of temperature and incubation time. It is worth noting that *B. ostreae* exhibited high survival under the full range of pH and temperature conditions tested. Besides, the tested range in temperature and pH is far greater than the actual changes in the variables predicted in (Huthnance *et al.,* 2016) for the end of this century, so it seems unlikely that the *Bonamia* parasite will be strongly negatively impacted by climate change in the near to medium term. Yet, specific research on the interactions between climate change effects and *Bonamia* is needed to test this hypothesis.

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

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

Although marine invertebrates lack the ability to develop pathogen specific antibodies, *O. edulis* from *Bonamia*-exposed populations have demonstrated more resistance or tolerance to the parasite than oysters from *Bonamia*-free populations (Hervio *et al.,* 1995; Culloty *et al.,* 2001; 2004; da Silva *et al.,* 2005). (Morga *et al.,* 2017) demonstrated a degree of disease resistance in *Bonamia*-exposed oysters, with inhibiting phagocytotic activity to reduce the spread of parasites to further tissue, while inducing in haemocytes the expression of genes associated with apoptosis, thus hampering parasite proliferation within haemocytes.

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

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

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

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

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

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

5.6 Biosecurity measures

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

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

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

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

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

5.8 Maintaining genetic diversity

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

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

6. Recommendations for restoration practice

6.1 Avoidance of spreading diseases in general

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

6.3. Recommendations for future research

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

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

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