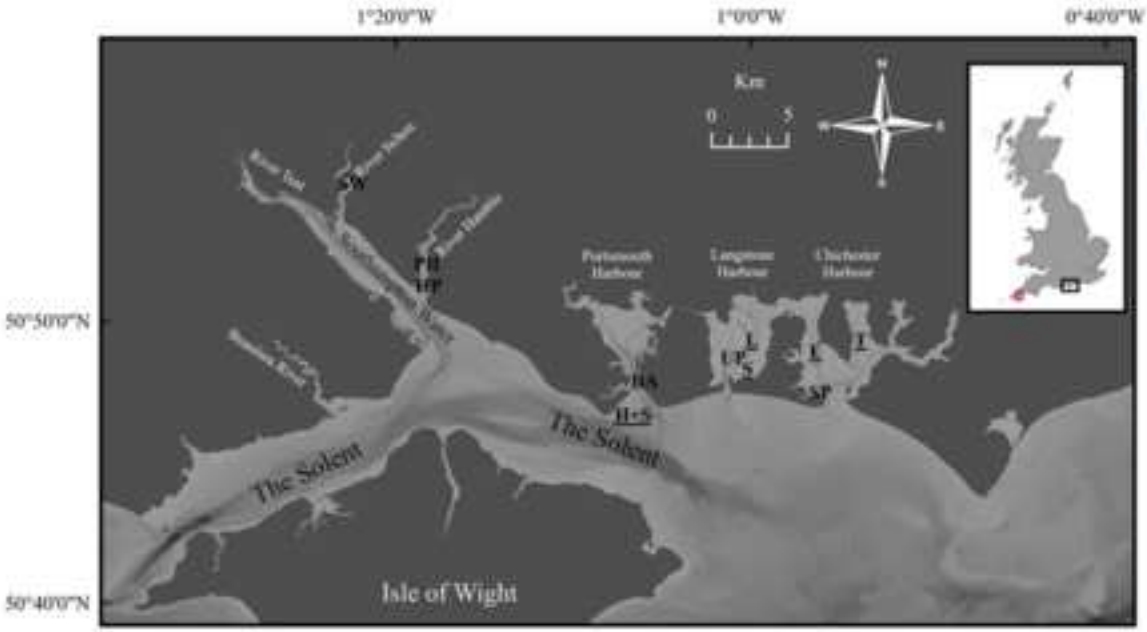
Ephemeral detection of Bonamia exitiosa (Haplosporida) in adult and larval European flat oysters Ostrea edulis in the Solent, United Kingdo

Highlights

- First molecular detection of *Bonamia exitiosa* in the Solent and second in the UK.
- 380 samples analysed with the pathogen observed in mature and larval *O. edulis*.
- Larvae shown to be positive were collected from brooding adults that were negative.
- Larvae should be considered as a possible source of *B. exitiosa* infection.
- Larvae should also be considered for disease resistance selection.



Adult (A):
B. exitiosa PCR -

Larvae (L):
B. exitiosa PCR +

Year	Location	n (Adult / Larvae)
2015	H+S	1 (A)
2017	PH	2 (A)
2017	BA	1 (A)
2017	UP	1 (L)
2017	SP	4 (L)
2018	SP	1 (L)

Possible introduction from New Zealand → **Europe**

Spain Atlantic	**Italy** Adriatic	**France / Spain** Mediterranean	**Portugal** Algarve **UK** Cornwall	**UK** Solent	**UK** Solent	**UK** Solent
2006	2007	2010	2013	2015	2017	2018

Title

Ephemeral detection of *Bonamia exitiosa* (Haplosporida) in adult and larval European flat oysters *Ostrea edulis* in the Solent, United Kingdom

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Abstract

The haplosporidian parasite *Bonamia exitiosa* was detected using PCR in four adult and six larval brood samples of the European flat oyster *Ostrea edulis* from the Solent, UK. This represents the second reported detection of this parasite along the south coast of England.

Adult oysters were collected and preserved from seabed populations or restoration broodstock cages between 2015 - 2018. The larvae within brooding adults sampled during 2017 and 2018 were also preserved. Molecular analysis of all samples was performed in 2019. The DNA of *B. exitiosa* was confirmed to be present within the gill tissue of one oyster within the Portsmouth wild fishery seabed population (n = 48), sampled in November 2015; the congeneric parasite *Bonamia ostreae* was not detected in this individual. This is the earliest record of *B. exitiosa* in the Solent. Concurrent presence of both *B. ostreae* and *B. exitiosa*, determined by DNA presence, was confirmed in the gill and heart tissue of three mature individuals from broodstock cages sampled in October 2017 (n = 99), two from a location on the River Hamble and one from the Camber Dock in Portsmouth Harbour. *B. exitiosa* was not detected in the November 2018 broodstock populations. A total of six larval broods were positive for *B. exitiosa*, with five also positive for *B. ostreae*. None of the brooding adults were positive for *B. exitiosa* suggesting that horizontal transmission from the surrounding environment to the brooding larvae is occurring. Further sampling of broodstock populations conducted by the Fish Health Inspectorate at the Centre for Environment, Fisheries and Aquaculture Science in June 2019 did not detect infection of *O. edulis* by *B. exitiosa*. These findings together suggest that the pathogen has not currently established in the area.

Key words

Oyster; *Ostrea edulis*; *Bonamia exitiosa*; *Bonamia ostreae*; Molecular diagnosis; haplosporida

1. Introduction

As efforts to restore the European flat oyster *Ostrea edulis* gain momentum across Europe, disease prevalence and resistance within populations will play a pivotal role in their

success. Of particular concern is the impact of European Commission (EC) notifiable protozoan parasites within the genus *Bonamia* (Haplosporidia; Sprague 1979), especially *B. ostreae*. The disease bonamiosis, caused by members of the genus of intrahaemocytic protozoan parasites *Bonamia*, including *Bonamia ostreae*, has severely impacted *O. edulis* populations. The microcells (2 - 5 µm diameter) of *B. ostreae* enter into the haemocytes of the oysters by host-specified phagocytosis (Chagot et al., 1992) and become systemic, overwhelming and eventually killing the infected individual. The distribution, spread and mass mortality events caused by *B. ostreae*, since its introduction to Europe in the 1970s and '80s (MacKenzie et al., 1997), are well documented (Figueras, 1991; Cigarria et al., 1995, Laing et al., 2005; Culloty and Mulcahy, 2007) with its impact as a non-native species driving disease emergence highlighted by Peeler et al. (2011).

Another member of the genus, *B. exitiosa,* first detected in the southern hemisphere in association with the host *Ostrea chilensis* (Dinamani et al., 1987; Cranfield et al., 1991; Hine et al., 2001) has subsequently been detected in *O. edulis* across continental Europe. The first detection occurred in 2006 (Galician coast, Spain (Abollo et al., 2008)), shortly followed by another in 2007 (Adriatic Sea, Italy (Narcisi et al., 2010)). The species has subsequently been detected in France (Mediterranean Sea (Arzul et al., 2010)), the Spanish Mediterranean coast (Carrasco et al., 2012), Britain (Cornwall (Longshaw et al., 2013)) and Portugal (Algarve (Batista et al., 2016)). The first UK positive population in which *B. exitiosa* was detected, was in the River Fal (Cornwall) (Longshaw et al., 2013), 28 years after the first diagnosis of *B. ostreae* in the UK, also in the River Fal (Bucke and Feist, 1985; Hudson and Hill 1991). To date there have been no reported mass mortality events in Europe where *B. exitiosa* has been considered the aetiological agent and a small number of infected individuals were detected within the sampled populations.

Bonamia exitiosa was first described infecting *Ostrea chilensis* in New Zealand (Hine et al., 2001), after a mass mortality event devastated an 'immunologically naïve' oyster population between 1986 and 1992 (Doonan et al., 1994; Cranfield et al., 2005). Retrospective analysis demonstrated that tissue samples infected with *B. exitiosa* date back to 1964, supporting the assumption that the species is endemic to New Zealand (at least) and that a relatively stable host/parasite relationship exists (Hine and Jones, 1994; Hine, 1996).

Unlike *B. ostreae*, where aspects of the life cycle remain unanswered (Culloty and Mulcahy, 2007), the life cycle of *B. exitiosa* is relatively well documented and is key to our understanding of dispersal mechanisms across a wide geographic range (Cranfield et al., 2005; Hill et al., 2014). The intrahaemocytic *B. exitiosa* spreads through the dispersal of infective particles released from the gonads, kidneys, gills and gut tissue of the diseased or dying oyster host (Hine, 1991a, 1991b). Once ingested by oysters in close proximity these new hosts become infected when the parasite enters the blood via the gut (Hine and Jones, 1994). There is currently no literature available describing the occurrence of vertical transmission from parent to larval brood, or of horizontal transmission in any of the host oyster species.

B. exitiosa is currently known to infect wild and aquaculture stocks of multiple oyster species from around the globe including *Ostrea chilensis*, *O. edulis, O. angasi, O. puelchana*, *O. stentina*, and *Saccostrea glomerata*, with at least occasional infection of *O. lurida*, *Crassostrea virginica* and *C. ariakensis* noted as well. The parasite has been associated with mass mortality events for some of these oyster species (Burreson et al., 2004; Corbeil et al., 2006; Hill et al., 2010; Kroeck, 2010; Carnegie et al., 2014; Hill et al., 2014; Engelsma et al., 2014).

We used molecular technology to document the presence of *B. exitiosa* and *B. ostreae* in *O. edulis* populations in the Solent, UK over multiple years.

2. Material and Methods

2.1. Oyster provenance

Oyster samples were collected within the Solent (the stretch of water separating Southern England from the Isle of Wight) between 2015 - 2018 for on-going monitoring conducted as part of the Solent Oyster Restoration Project (www.bluemarinefoundation.com/project/solent/). These samples were stored in 98% ethanol and were held at 4°C at the Institute of Marine Sciences (University of Portsmouth, Portsmouth, UK) until retrospective screening was conducted in 2019 for pathogen presence. In November 2015, oysters were collected from the seabed using a commissioned dredge fisher in the area managed by the Southern and Sussex Inshore Fisheries and Conservation Authorities, as described in Helmer et al. (2019). A sub-sample of these from Chichester Harbour (n = 48) and Portsmouth Harbour (n = 48) (locations H+S and E and T, respectively, Fig. 1) were sampled immediately and stored for later molecular analysis of pathogen DNA presence. The remaining oysters sourced from the fishery were translocated into restoration broodstock cages suspended from existing floating structures in Portsmouth Harbour - BA (individuals from H+S) and Langstone Harbour - UP (individuals from E and T) in December 2015 (Fig. 1). Additional oysters (BA n = 42, UP n = 16) were sampled from these cages in July 2016 and stored as above.

Oysters sampled in October 2017 (n = 99) and November 2018 (n = 70) were originally purchased from the catch of the 2016 dredge fishery in Langstone Harbour (Locations L and S, Fig. 1). The 2016 seabed oysters were translocated into broodstock cages at two marina locations in the River Hamble in November 2016 (PH and HP, Fig. 1), and then distributed to

four additional locations across the Solent in March 2017 (SW, BA, UP and SP, Fig. 1, in addition to PH and HP). Oyster samples were taken and preserved from all marina locations during October 2017 and November 2018. Oysters collected in 2017 and 2018 were monitored for the presence of larvae within the pallial cavity and the white, grey or black "sick" larval stage was also recorded (Fig. 2). Brooding adults, and their larvae, were sampled and preserved for later molecular analysis. Adult gill and heart tissues were stored separately from the larval brood; a 250-µl aliquot of each brood was preserved in ethanol. A total of 35 broods were analysed, 31 from 2017 and 4 from 2018, with 21 of these having been sampled from brooding adults that were also screened for *B. exitiosa*.

The Fish Health Inspectorate (FHI) of England and Wales was contacted immediately upon PCR detection of *B. exitiosa*. Upon suspicion of presence of this exotic pathogen, the FHI carried out statutory sampling of *O. edulis* populations to test for the presence of *B. exitiosa*: 129 oysters were sampled from Port Hamble Marina (PH, Fig. 1), along with 150 from the Camber Dock, Portsmouth Harbour (BA Fig. 1) in March 2019, and an additional 26 oysters were sampled from the University of Portsmouth research platform in Langstone Channel (UP, Fig. 1) in June 2019. Tissue 'steaks' were dissected from each oyster and fixed for histopathology and molecular analyses and were processed for both methods as described in Longshaw et al. (2013).

2.2. Genomic DNA extraction and PCR amplification

A 5-mm section of gill tissue and the whole heart from each of the adult 2017 brooding, 2017 broodstock and 2018 broodstock samples were removed and stored in 98% ethanol before maceration with a sterile scalpel or pellet pestle. A 5-mm section of gill tissue was analysed from the 2015 seabed and 2016 broodstock samples, and was also removed and stored in 98% ethanol prior to maceration. The larval broods, rinsed with 0.2 µm filtered

seawater and 98% ethanol prior to storage in 98% ethanol, required no mechanical breakdown for the extraction process. All DNA extractions were performed using DNeasy® Blood & Tissue kits (QIAGEN™) following the manufacturer's tissue protocol. Quantification of DNA was conducted using a NanoDrop® 1000 Spectrophotometer (NanoDrop®, Thermo Fisher Scientific Inc., Wilmington, USA).

The *Ostrea edulis* species-specific primer pair Oe fw_1 + Oe rev_4 (5'-ATG-GGA-CGA-TTT-GAT-AGA-GC-3' and 5'-CCC-AAA-TAA-CGG-GAA-AAG-TGC-TAA-CCA-CCA-GAA-TGA-3', respectively) (Gercken and Schmidt, 2014) was used to amplify the cytochrome c oxidase subunit I (COI) gene from *O. edulis* as a positive control for oyster species confirmation. Due to the potential for concurrent infection of both *B. ostreae* and *B. exitiosa* and the specificity of OIE recommended primer pairs for *B. ostreae* over *B. exitiosa* (Helmer et al. unpublished results), the species-specific primer pairs BOSTRE-F + BOSTRE-R (5'-TTA-CGT-CCC-TGC-CCT-TTG-TA-3' and 5'-TCG-CGG-TTG-AAT-TTT-ATC-GT -3', respectively) (Ramilo et al., 2013) and BEXIT-F + BEXIT-R (5'-GCG-CGT-TCT-TAG-AAG-CTT-TG-3'and 5'-AAG-ATT-GAT-GTC-GGC-ATG-TCT-3', respectively) (Ramilo et al., 2013) were used to amplify the 18S-ITS1 rRNA gene region present from *B. ostreae* and *B. exitiosa*, respectively. The OIE recommended 18S primer pair BO + BOAS (5'-CAT-TTA-ATT-GGT-CGG-GCC-GC-3' and 5'-CTG-ATC-GTC-TTC-GAT-CCC-CC-3', respectively) (Cochennec et al., 2000) was also used to amplify *B. ostreae* DNA. Polymerase chain reaction (PCR) amplifications consisted of 12.5 μl 2 x DreamTaq™ PCR Master Mix (Thermo Fisher Scientific Inc.) or 12.5 μl 2 x DreamTaq Green PCR Master Mix (Thermo Fisher Scientific Inc.), 0.2 μM forward and reverse primers (Invitrogen, Thermo Fisher Scientific Inc.) and 20 - 200 ng genomic DNA made up to a final volume of 25 μl with molecular biology grade water. A negative control, with molecular biology grade water in place of template DNA, was run alongside each reaction. No negative controls amplified

during the course of the current study. No positive control was available at the outset. *B. exitiosa*-positive PCR products generated using the BEXIT primer pair during earlier sampling in the current study, later confirmed by sequencing of the 18S-ITS1 gene region, were used as positive controls in the latter PCR analysis.

PCRs were run in a G-STORM 482 Thermal Cycler (Gene Technologies Ltd., Essex, England) under the respective conditions described by Cochennec et al. (2000), Ramilo et al. (2013), Gercken and Schmidt (2014). PCR products were separated on 1% (Oe) or 2% (BOSTRE, BEXIT and BO + BOAS) 1x TAE (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) agarose gels stained with 4 ul ethidium bromide. Electrophoresis ran at 100 V for 1 h. A 1-kb GeneRuler™ DNA ladder (Thermo Fisher Scientific Inc.) or 100-bp DNA ladder (New England Biolabs® or PCR Biosystems Ltd) and PCR products were visualized by ultraviolet (UV) transillumination (VWR Gel Documentation Smart Version).

PCR products of all *B. exitiosa*-positive amplifications using the BEXIT-F + BEXIT-R primer pair and reference samples and strong bands from Oe fw_1 + Oe rev_4, BOSTRE-F + BOSTRE-R and BO + BOAS primer pairs were purified using a QIAquick® PCR Purification Kit (Qiagen) following the manufacturer's protocol. Amplicons were sequenced by Sanger sequencing (Source BioScience, Nottingham, England) using the respective primer pairs used for PCR, and the electropherograms analysed by eye in MEGA X (Pennsylvania State University, USA). Where possible, contigs were assembled using CAP3 sequence assembly program (Huang & Madan, 1999). The resulting contig sequences were BLASTn® -searched against the nr/nt database of the National Center for Biotechnology Information web server. Sequences were deposited into GenBank (Accession numbers MT184259 - MT184268).

3. Results

Of the 96 oysters sampled from the 2015 Seabed populations, one individual (1.04%), from the Portsmouth fishery area (H+S Fig. 1), was positive for *B. exitiosa* based on the amplification of the expected 246 bp using the BEXIT-F + BEXIT-R primer pair. The sequence of the PCR-amplification product showed 99.59% identity to a *B. exitiosa* sequence from Tunisia (JF831718.1). *B. ostreae* was not detected in this individual using the BOSTRE-F + BOSTRE-R primer pair. *B. ostreae* DNA was detected in 34.4% and 49% of the oysters using the BO + BOAS and BOSTRE primer pairs, respectively.

Of the broodstock oysters sampled in 2017, three (3.03%) screened positive for *B. exitiosa* DNA. Of those, two were located in the same marina on the River Hamble (PH, Fig. 1) and the other in Portsmouth Harbour (BA, Fig. 1). The sequence of the PCR-amplification products from the River Hamble showed 100% identity to a *B. exitiosa* sequence from North Carolina (JF831588.1), whilst the sample from Portsmouth Harbour showed 100% identity to a *B. exitiosa* isolate sequence from Australia (JF831683.1). Both oysters from the River Hamble and the individual from Portsmouth Harbour were also positive for *B. ostreae* DNA, with a 208-bp amplicon from the BOSTRE-F + BOSTRE-R primer pair. *B. ostreae* DNA was detected in 85.7% and 98% of the oysters using the BO + BOAS and BOSTRE primer pairs, respectively. No oysters sampled from the 2016 or 2018 broodstock cages tested positive for *B. exitiosa*. However, in 2016, 34.5% and 46.6% of oysters tested PCR-positive for *B. ostreae* using the BO + BOAS and BOSTRE primer pairs, respectively. In 2018, the same primer pairs resulted in 54.3 % and 81.4 % of oysters also testing PCR-positive for *B. ostreae.*

Of the 10 larval broods analysed without the respective adult collected for analysis, none were PCR-positive for *B. exitiosa*. A total of 21 brooding adults and their larval broods from 2017 were analysed; none of the brooding adult oysters tested positive for *B. exitiosa* (Fig. 3). The larval brood from one PCR-negative adult oyster in Chichester Harbour tested

positive using PCR and showed 100% identity to a *B. exitiosa* sequence from North Carolina (JF831588.1). Another four broods were PCR-positive with the BEXIT primers, but the F and R sequences did not form a contiguous sequence due to either low sequence quality or lack of consensus. The latter could be due to multiple parasites occurring within the brood. Further work is required to clarify the validity of these results. Of the four broods collected from Chichester Harbour in 2018, one provided a sequence contig that showed 100% identity to a *B. exitiosa* sequence from North Carolina (JF831588.1). The positive results obtained for all adult and larval samples are summarised in Table 1 and the sample groupings from all years in Table 2. In 2017, 64.5% and 77.4% of the larvae tested PCR-positive for *B. ostreae* using the BO + BOAS and BOSTRE primer pairs, respectively. This was also the case for 50% and 75% of larval samples in 2018, using the same respective primer pairs. Sequences with a similarity to *B. exitiosa* greater than 98% were submitted to GenBank (Accession numbers MT184259 - MT184268).

Table 1. Details of samples that tested positive by PCR screening for *Bonamia exitiosa* with highest sequence identity from GenBank BLASTn search. Samples with no contiguous sequence that provide tentative results are grouped with respective borderlines and grey scaled, F or R denotes the forward or reverse primer sequence used.

Study sample information			GenBank search results				
Location in Figure 1	**Sample group**	**Sequence**	**Top GenBank match**	**Identity %**	**Geographic region**	**Host species**	**GenBank accession**

H+S	2015 Seabed	Contig	*B. exitiosa*	99.59	Tunisia	*Ostrea stentina*	JF831718
Port Hamble	2017 Broodstock	Contig	*B. exitiosa*	99.18	North Carolina	*Ostrea stentina*	JF831588
Port Hamble	2017 Broodstock	Contig	*B. exitiosa*	100.00	North Carolina	*Ostrea stentina*	JF831588
Portsmouth	2017 Broodstock	Contig	*B. exitiosa*	100.00	Australia	*Saccostrea glomerata*	JF831683
Chichester	2017 Larvae	Contig	*B. exitiosa*	100.00	North Carolina	*Ostrea stentina*	JF831588
Chichester	2018 Larvae	Contig	*B. exitiosa*	100.00	North Carolina	*Ostrea stentina*	JF831588
Langstone	2017 Larvae	F	*B. exitiosa*	98.59	Argentina	*Ostrea stentina*	JF831559
Langstone	2017 Larvae	R	*B. exitiosa*	100.00	Tunisia	*Ostrea stentina*	JF831718
Chichester	2017 Larvae	F	*B. exitiosa*	97.77	New Zealand	*Ostrea chilensis*	KY680634
Chichester	2017 Larvae	R	N/A	N/A	N/A	N/A	N/A
Chichester	2017 Larvae	F	N/A	N/A	N/A	N/A	N/A
Chichester	2017 Larvae	R	*B. exitiosa*	100.00	Australia (NSW)	*Saccostrea glomerata*	JX977122
Chichester	2017 Larvae	F	*B. exitiosa*	93.70	California	*Ostrea conchaphila*	JF831733
Chichester	2017 Larvae	R	*B. exitiosa*	98.40	Tunisia	*Ostrea stentina*	JF831718

Table 2. Summary of sample populations, sample type, number of oysters from each location and population sampled. Bold numbers in parentheses indicate the number of PCR-positive *Bonamia exitiosa* samples from the respective sample set obtained using high quality consensus sequence reads. Numbers not in bold indicate those samples where identification requires further analysis.

	Number of oysters per location				
River Itchen	**River Hamble**	**Portsmouth Harbour**	**Langstone Harbour**	**Chichester Harbour**	**Total**

Sampling year	Sample type	SW	PH	HP	H+S	BA	UP	E / T	SP	
2015 Seabed populations	Gill				48 **(1)**			48		96
2016 Broodstock cages	Gill					42	16			58
2017 Broodstock cages	Gill + Heart	17	17 **(2)**	17		17 **(1)**	17		14	99
2017 Brooding individuals (within cages)	Gill + Heart	1	3	3		2	5		8	22
2017 Larvae	Larvae	2	4	4		4	8 **(1)**		9 **(1,3)**	31
2018 Broodstock cages	Gill + Heart	10	12	12		12	12		12	70
2018 Larvae	Larvae								4 **(1)**	4

The 305 samples collected by the Fish Health Inspectorate (FHI) of England and Wales and analysed by the Statutory Diagnostic Team at the Centre for Environment, Fisheries and Aquaculture Sciences (CEFAS) were all PCR-negative for *B. exitiosa* using the lineage-specific BEXIT primers. *B. exitiosa* was not observed in any histology screens of the animals.

4. Discussion

The current study describes the second detection of *B. exitiosa* in *O. edulis* in the UK, with concurrent detection of *B. ostreae*, as previously reported by Abollo et al. (2018), Ramilo et al. (2014) and Lane et al. (2016). It should be noted that not all the samples in the present study were analysed by histology or heart smears, therefore only the DNA of *B.*

exitiosa and *B. ostreae* was detected for a proportion of the population in this study. Infection of native oysters by *B. exitiosa* was not confirmed by microscopic examination or histology because no diseased oysters were observed; therefore, the possibility the pathogen was dormant or not viable cannot be ruled out (Burreson, 2008). The detection of *B. exitiosa* was ephemeral in nature and limited to a small portion of the populations monitored and no disease symptoms or mortality was attributed to *B. exitiosa*. Mortality experienced within the monitored populations is more likely attributed to a combination of post-spawning mortality (Helmer, unpublished data), environmental stressors, such as temperature and salinity, and the high prevalence of *B. ostreae* observed. As *B. ostreae* is well established in the area (Laing et al., 2014) it is unsurprising that such high proportions of the oysters sampled tested PCR-positive for this pathogen.

Despite the lack of histological analysis to indicate infection intensity by *B. exitiosa* in this case, and the lack of DNA detection during statutory disease assessments of oysters sampled from two proximal sites in 2016, 2018 and 2019, the distribution and potential impacts of *B. exitiosa* across Europe requires further investigation. In addition, a detailed investigation into the phylogeny and origin of the strains for members of the *Bonamia* genus in *O. edulis* populations is strongly recommended, as the complete status across Europe is currently unknown, even though the presence of *B. ostreae* is relatively well documented.

The only other characterised *Bonamia* species is *B. perspora* (Carnegie et al., 2006). It is believed that *B. perspora* is host specialist and currently maintains a well-defined and restricted geographical range infecting *Ostrea stentina* in North Carolina (Carnegie et al., 2006; Hill et al., 2014), thus unlikely to be present in *O. edulis* within Europe. Infections of *B. perspora* were not observed in populations of *O. stentina* in Argentina, Tunisia and New Zealand (Hill et al., 2014) but its presence in *O. stentina* in areas across Europe remains untested. *Bonamia roughleyi* was first described as a distinct species (Cochennec-Laureau et

al., 2003), but Carnegie et al. (2014) questioned its identity, arguing that there is a lack of genetic distinction between *B. exitiosa* and *B. roughleyi*.

To date there have been no reported mass mortalities of *O. edulis* within Europe where *B. exitiosa* has been identified as the aetiological agent; all accounts have reported *B. ostreae* to be the responsible pathogen. All reported detections of *B. exitiosa* in *O. edulis* have been in a small proportion of the tested populations, with Abollo et al. (2008) reporting the highest prevalence of 40.2% with 16.5% co-infection with *B. ostreae*. Batista et al. (2016) reported positives in 83.3% of samples but the small sample size was small (n = 24). In many cases co-infection with *B. ostreae* was reported; we found only one adult and one larval brood infected with *B. exitiosa* but not *B. ostreae*.

The ability of *O. edulis* to tolerate co-infection with *B. exitiosa* and *B. ostreae* may be due to the similarity of the two pathogen species but also their difference in lethality, with the 18-week 50% lethal dose of *B. ostreae* in *O. edulis* (Hervio et al., 1995) being 40% lower than that of *B. exitiosa* in *O. chilensis*, the former determined to be ~ 1.1×10^5 infective particles (Diggles and Hine, 2002). This indication that *B. ostreae* is far more virulent than *B. exitiosa* suggests that any resistance, tolerance or resilience to *B. ostreae* within European populations of *O. edulis*, developed in the 30-40 years since its introduction (1970s - 80s) (MacKenzie et al., 1997; Culloty and Mulchay, 2007; Lynch et al., 2014), may provide a level of resistance, tolerance or resilience to *B. exitiosa* that impedes its rapid proliferation. Another possibility is that interspecific competition between the two pathogens is occurring, with *B. ostreae* excluding or outcompeting *B. exitiosa*. Such interactions are yet to be investigated in these species.

The detection of *B. exitiosa* has implications for management of infected populations as this pathogen is included, along with *B. ostreae*, within the list of notifiable species by the

World Organisation for Animal Health (OIE) (http://www.oie.int/animal-health-in-the-world/oie-listed-diseases-2019/, last accessed 26 March 2019) and the EC Council Directive 2006/88/EC (https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2006:328:0014:0056:en:PDF%20, last accessed 18 May 2019) with this document legally ensuring that the Fish Health Inspectorate of England and Wales, the responsible entity for fish and shellfish health, regularly monitor for *B. ostreae* and *B exitiosa*.

The increase in geographical distribution of *B. exitiosa* (Hill et al., 2014) is likely to be attributed to its dispersal potential. Survival of infective particles in seawater has been shown to be 50% after 48 h at 18ºC (Diggles and Hine, 2002) and detection of *B. exitiosa* in *O. edulis* larvae (Arzul et al., 2011) suggests that if the pathogen is viable in, or incidentally attached to the larvae, dispersal or spread could be accelerated. The detection of *B. exitiosa* in larval broods of PCR-negative adults indicates the occurrence of horizontal transmission by release of the pathogens from dead or dying oysters (Hine 1991a, b; Audemard et al., 2014) or other vector taxa. Evidence of horizontal transmission has been described for *B. ostreae* in *O. edulis* (Arzul et al., 2011; Flannery et al., 2016), with Lynch et al. (2010) also detecting *B. ostreae* DNA in the pallial fluid. The capacity of *O.edulis* larvae to feed within the pallial cavity during the brooding period (Hine and Jones, 1994; Helm et al., 2006) and detection of *B. ostreae* in the epithelia surrounding the visceral cavity of infected larvae (Arzul et al., 2011), further highlights the opportunity for transmission of *B. exitiosa* in this manner. This mounting evidence of larval infection highlights one of many potential transmission pathways of *Bonamia* infection to naïve oyster populations, with larvae having been shown to travel up to 12 km from the source location (Wilson, 1987).

Restoration efforts, whereby oysters are transported into areas that have been left fallow for prolonged periods, should continue to include monitoring for both *B. ostreae* and *B.*

exitiosa. van Banning (1998) showed that *B. ostreae* can persist in the environment in the absence of *O. edulis* and infect newly introduced naïve oysters, with Lynch et al. (2007) suggesting that this could be due to the potential of multiple macroinvertebrate species to act as carriers. All eight potential carrier species observed in that study, including *Actinia equine*, *Carcinus maenas* and *Ascidiella aspersa*, are present and abundant within the Solent and across much of Europe. The presence of *B. ostreae* in the 2007 study was not limited to benthic species - grouped zooplankton species also yielded positive results adding to the potential for vectoring. For example, the copepod *Paracartia grani* acts as an intermediate host for another serious oyster parasite, *Marteilia refringens* (Audemard et al., 2002). The ability of *B. exitiosa* to utilise intermediate hosts is currently unknown and also requires further research.

Evidence that the Pacific oyster *Crassostrea gigas* may be a host for both *B. ostreae* and *B. exitiosa* (Lynch et al., 2010; Flack, unpublished results) is of particular concern as the species' distribution and abundance across Europe has increased in recent years (Anglès d'Auriac et al. 2017). This potentially provides "stepping-stones" for disease transfer between remaining populations of *O. edulis* that are currently fragmented. Further clarity of the disease vector role played by *C. gigas* is required for areas across Europe where it is present in aquaculture or wild populations. Confirmation of *C. gigas* as a disease vector may require the active management and removal of significant populations in order to aid prevention of disease transmission within flat oyster populations. Alternatively, Pacific oysters could be paratenic or dead-end hosts acting as sinks for the pathogens, indefinitely or until prevalence reaches a threshold. Similarly, the role of disease transmission by the invasive and highly abundant American slipper limpet *Crepidula fornicata* (Helmer et al., 2019) is unclear and needs to be determined, with the potential of additional supporting information to justify their removal on a large scale.

Incidents of *B. exitiosa* infecting *O. edulis* where *B. ostreae* is not present have been observed previously (Batista et al., 2016). The first European detection of *B. exitiosa* on the Galician coast in 2006, followed by infections along the French Atlantic coast in 2008 and then the River Fal in 2010, suggests that a combination of anthropogenic oyster movements, larval dispersal and infective particle transmission enables the relatively rapid transmission of *B. exitiosa* north-eastward. The ability of *Bonamia* species to parasitize a range of hosts, alongside infective particle dispersal and larval infection, is likely to have contributed to its dispersal on a global scale, including New Zealand, Australia and Argentina in the southern hemisphere, and Atlantic coastlines (US and Europe), Pacific coastlines (US) Mediterranean Sea and English Channel in the northern hemisphere.

It is uncertain if the presence of *B. exitiosa* poses a threat to progress made with the selective breeding for resistance to *B. ostreae* in the European flat oyster (Hervio et al., 1995; Culloty et al., 2004; Lynch et al., 2014). Mortality events of *O. edulis* should continue to be monitored rigorously, as the species is unlikely to fare well with the introduction of this additional non-native protozoan if it reaches a significant prevalence within a population. The impact of such an event can be seen from the mass mortalities in Europe induced by the initial introduction of *B. ostreae* from the west coast of the USA (Elston et al., 1986; MacKenzie et al., 1997). It is therefore recommended that monitoring for, and restricted movement of, oysters infected with *B. exitiosa* be incorporated into section 4 (Respect *Bonamia*-free areas) of the Berlin Oyster Recommendations (Pogoda et al., 2019). These recommendations were compiled through a collaborative assessment of the current European restoration efforts with the aim of developing and sharing best restoration practices for the species, a prime example of information sharing.

For *O. edulis* restoration efforts to be successful, as they have been for other oyster restoration projects in disease-stricken sites around the world, (Proestou et al., 2016),

deploying large quantities of oysters in high-density populations will be required to recreate a fraction of historical population densities. This may incur significant mortality due to disease, but assuming the stocks used are genetically robust and diverse it also provides an opportunity for natural resistance to develop over time.

5. Conclusion

The low levels of detection of *B. exitiosa*, along with no increase in detection in high-density oyster populations over subsequent years, suggests the parasite has either failed to establish in the Solent or may have established at low enzootic levels. However, the current study highlights the risk of emerging and known pathogens to oyster restoration and aquaculture in Europe and further emphasises the requirement for continued control of oyster translocation. Biosecurity controls are the only method currently available to prevent or postpone the spread of *Bonamia* parasites, but as can be observed by the continued spread of pathogens, and as reported here, these control measures are not always successful. Therefore, it is clear that further research is required fully understand the mechanism of transmission, the vector species and environmental pathways through which *Bonamia* pathogens enter previously disease-free sites in order to successfully manage bonamiosis.

Ethical approval

All applicable international, national and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participant performed by any of the authors.

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Figure

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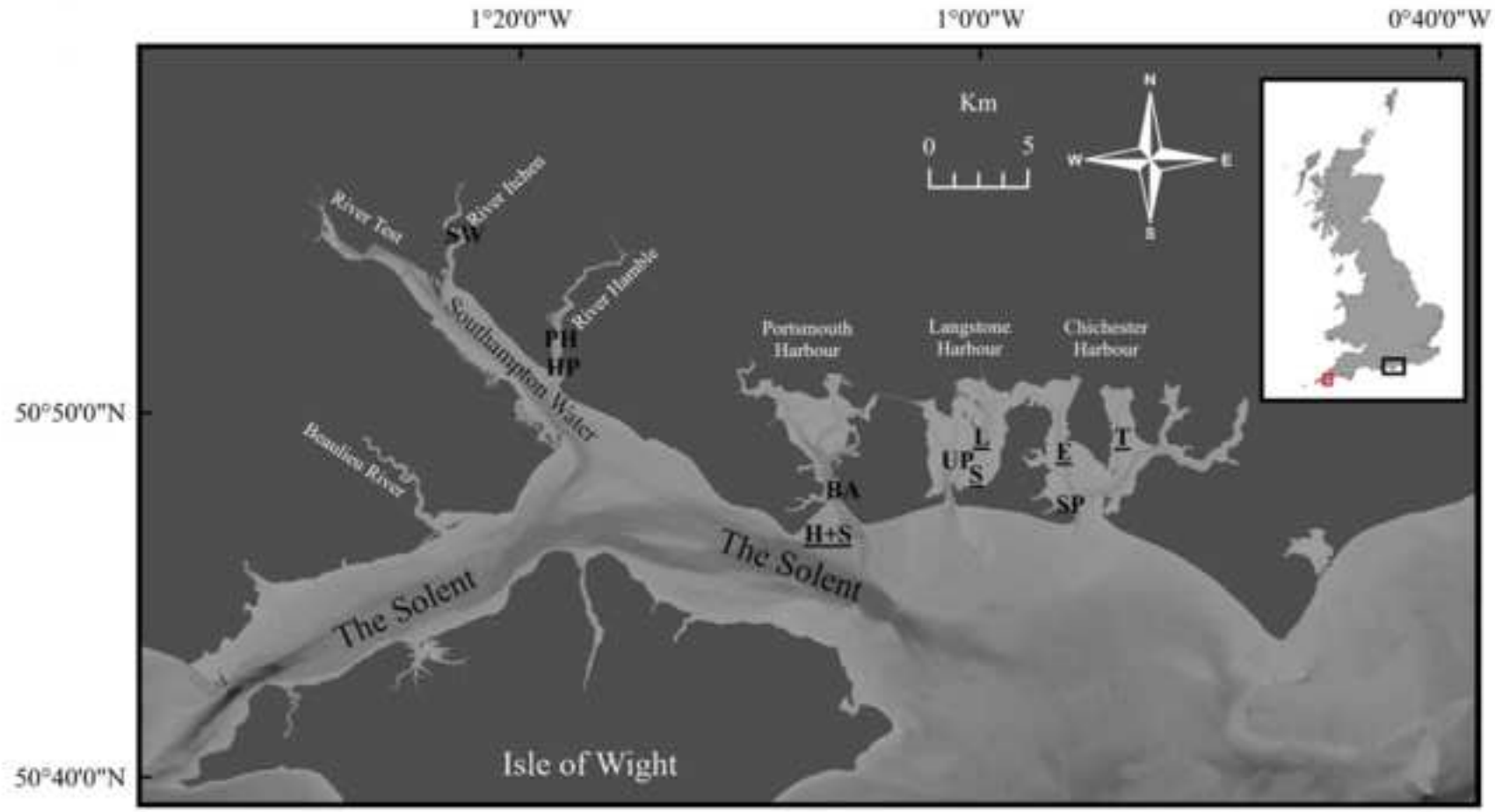

Figure

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