Journal of Invertebrate Pathology 118 (2014) 59–65

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

The health status of mussels, *Mytilus* spp., in Ireland and Wales with the molecular identification of a previously undescribed haplosporidian

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ARTICLE INFO

Article history:
Received 22 October 2013
Accepted 27 February 2014
Available online 6 March 2014

Keywords:
Mytilus spp.
Hybrids
Health
Parasites
Environmental drivers

ABSTRACT

Both wild and cultured mussels (*Mytilus edulis*, *Mytilus galloprovincialis* and hybrids), are found along most of the Irish coastline. *M. edulis* is widespread along all Irish coasts and is the only mussel species present on both the east coast of Ireland and the Welsh coast in the Irish Sea. *M. galloprovincialis* and hybrids are found along the Irish coastline except for the east coast. Samples of *Mytilus* spp. were collected from twenty-four sites, encompassing all coasts of Ireland and the Welsh coast, at different times of the year and over several years (2008–2011). In total, 841 mussels were examined histologically to assess their health status and the presence of any parasites or commensals. Mussels from 14 of the 24 sites were screened using polymerase chain reaction (PCR) to determine which mytilid species were present. A range of parasites were observed, generally at low levels. The most diverse community of parasites was observed at a sheltered site with poor water quality. Of significance, a previously undescribed haplosporidian was detected in a single mussel sample in the Menai Strait, Wales, by PCR and was confirmed by direct sequencing and is most closely related to *Minchina chitonis* and a haplosporidian of the Florida marsh clam *Cyrenoida floridana*. While *M. edulis* were infected by a variety of micro- and macro-parasites, only trematodes were observed in *M. galloprovincialis* and hybrids. Habitat description and the environmental factors influencing the study sites, including water quality and exposure, were recorded.
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1. Introduction

Mytilus edulis are boreo-temperate in their distribution on both coasts of the Atlantic Ocean and are found in abundance, intertidally and subtidally, in both sheltered and exposed sites, attached to hard substrates or forming biogenic reefs. In the western Atlantic, *M. edulis* is historically found from the Arctic Sea, Canada (Dall, 1889) to North Carolina, United States (Stimpson, 1860: McDougall, 1943) and in the eastern Atlantic occurs from Norway (Christiansen, 1965) to the border of France and Spain (Sanjuan et al., 1994). *Mytilus galloprovincialis* is endemic to the Mediterranean, Black Sea and Adriatic Sea and has expanded its range to the British Isles (Gosling, 1992). Evidence of hybridisation and hybrid zones of *M. edulis* and *M. galloprovincialis* in the south west of England and Ireland were first recorded in the 1970s and subsequent studies have further documented this phenomenon (Gosling and Wilkins, 1977; Skibinski et al., 1983; Gosling and McGrath, 1990; Gardner, 1997; Gosling et al., 2008). Hybrids are commonly thought to have lower fitness (Mayr, 1963), however, certain studies have shown "hybrid vigour" (heterosis) in the first hybrid generation (F1 hybrid) which may allow hybrids to function over a wider range of environmental conditions than the two parental species (Littlejohn and Watson, 1985). Both mytilid species are important commercially in Europe and mussel seed is collected from wild beds or collector ropes and transferred to other areas for on-growing (Fuentes et al., 2002; Kijewski et al., 2009). In Europe, *M. edulis* is harvested extensively from both wild and farmed sources while *M. galloprovincialis* is harvested from cultured stock.

Parasites of mussels can have a detrimental effect on both natural and cultured stocks (Sindermann and Rosenfield, 1967) and it is acknowledged that the prevalence of parasites, mussel condition index and associated mussel mortalities should be examined more

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http://dx.doi.org/10.1016/j.jip.2014.02.012
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extensively (http://www.fao.org/fishery/culturedspecies/Mytilus_galloprovincialis/en). Environmental factors such as poor water quality and the presence of parasites have been shown to have had an impact on the health status of mussel stocks in Sweden and the UK (Svärdh, 2003; Bignell et al., 2008). A decrease in water quality can affect the immunological response in aquatic organisms thus making them more susceptible to parasitic infection and increasing parasite prevalence (Khan and Thulin, 1991). Organic pollution is associated with a decrease in dissolved oxygen, which creates a favourable environment for bacteria, while inert suspended solids can damage the gill epethelium and make individuals more susceptible to infection with fungi, etc. (Svobodova et al., 1993). Mussels are commonly used as environmental indicators based on surrounding water quality, which may in turn impact on their susceptibility to disease and parasites.

It has been suggested that high population densities associated with aquaculture may trigger a disease outbreak (Morley, 2010). Several parasites and pathogens have been identified in *M. edulis* and *M. galloprovincialis* (Bayne, 1976; Paul, 1983; Bower et al., 1994; Gosling, 1992; Calvo-Ugarteburu and McQuaid, 1998; Bower et al., 1994; Le Roux et al., 2001; da Silva et al., 2002; Ciacci et al., 2009). The two individual mytilid species, together with their hybrids, are considered to be susceptible to a similar diversity of pathogens (Bignell et al., 2008). A recent study investigating the occurrence of macroparasites in several common intertidal molluscs on the south coast of Ireland detected four trematode species in *Mytilus* spp. and the copepod *M. intestinalis* (Prinz et al., 2010).

The objectives of this study were (a) to investigate the health status of mussels around the entire coast of Ireland and Wales, encompassing both the Atlantic coast and the Irish Sea area, (b) identify the range of parasites and pathogens present at the individual level at different sites, (c) determine if any apparent difference in susceptibility to parasites exists between each mytilid species or hybrids and (d) examine the effect of site-related environmental factors, such as water quality and site exposure on health status.

2. Materials and methods

2.1. Sampling

In this study, a total of 751 mussels from both wild and cultured stocks were sampled from a total of twenty-one sites (including four sites within Cork Harbour on the south coast of Ireland) on all Irish coasts and three sites on the Welsh coast (two in north Wales and one in south Wales), at different times, encompassing all seasons from November 2008 to July 2011. The mussels were collected with sample sizes varying from 14 to 60 individuals. All wild mussels were collected from the intertidal zone and cultured mussels were sampled subtidally (Fig. 1 and Table 1).

A water quality classification system obtained from the Environmental Protection Agency (http://www.epa.i.e./water/watmg/wfd/classification/) was applied to the different study sites based on anthropogenic inputs such as agricultural run-off, leachate from landfills and contaminated sites, untreated waste water and sewage discharge, increased recreational and boating use and industrial run-off in the surrounding catchment area at each site: Class A (very little if any anthropogenic effects), Class B (some anthropogenic effects) and Class C (site influenced greatly by anthropogenic effects).

2.2. Histology

A transverse section of mussel tissue (~1 cm^2) containing mantle, gill, digestive gland and gonad was excised and fixed in Davidson's fixative at 4 °C for 48 h (Shaw and Battle, 1957) before being transferred to 70% ethanol. The fixed tissue was then dehydrated through an ascending ethanol series and embedded in paraffin wax. A 5 μm tissue section was stained using haematoxylin and eosin. Sections were examined at 40× and at 100× under oil.

2.3. DNA extraction and standard polymerase chain reaction (PCR) for *Mytilus* species identification

Gill tissue was excised from 365 individual mussels from 11 of the 21 Irish sites and 90 mussels (from all 3 sites) in Wales and was stored in 96% ethanol (Table 2). DNA was extracted using the chelex-100 extraction method (Walsh et al., 1991; Lynch et al., 2008). A PCR was carried out to amplify the nuclear DNA markers Me15/Me16 to differentiate which *Mytilus* species or hybrid was being screened (Inoue et al., 1995). The PCR mastermix was modified slightly to include 5x green buffer. Amplification was conducted in 25 μl of the reaction mixture containing 14 μl ddH_2O, 5 μl 5× green buffer (Promegal), 2.5 μl of each of the four deoxyribonucleotide triphosphates (dATP, dCTP, dGTP, dTTP), 1.5 μl $MgCl_2$, 0.5 μl

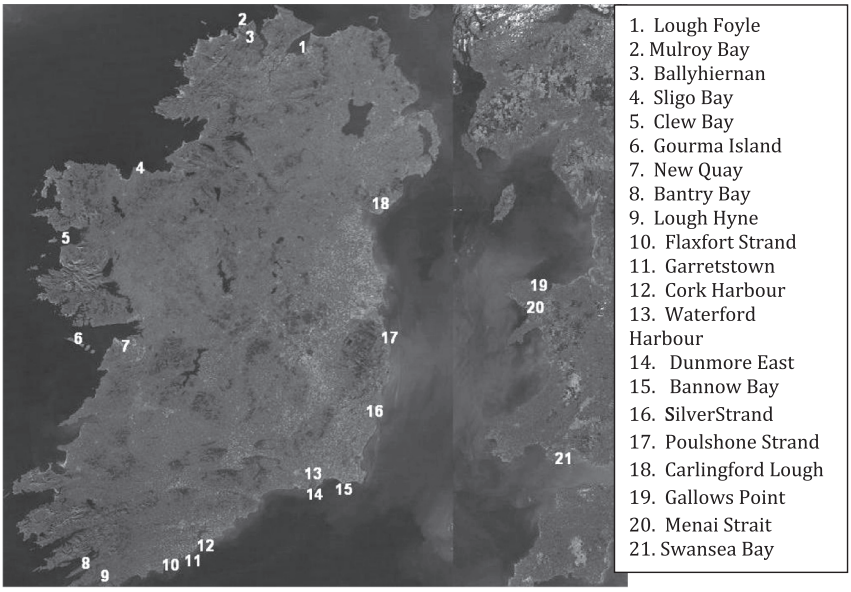

Fig. 1. Map of Ireland and Wales showing *Mytilus* spp. sampling sites.

Table 1
List of *Mytilus* spp. samples, description of sample and water quality at site.

Site	Grid reference	Sample date	Wild/cultured	*n*	Water quality
Ireland					
South coast					
Dunmore East (E)	52°08′N, 6°59′W	February 2011	Wild	30	Class B
Woodstown Beach, (E)	52°11′N, 6°58′W	April 2010	Wild	30	Class A
Bannow Bay (S)	52°13′N, 6°47′W	March 2010	Wild	30	Class B
Roches Point, Cork Harbour (E)	51°48′N, 8°15′W	June 2010	Wild	30	Class B
Whitegate, Cork Harbour (SE)	51°49′N, 8°13′W	June 2010	Wild	30	Class C
Cobh, Cork Harbour (S)	51°50′N, 8°18′W	June 2010	Wild	30	Class C
Ringaskiddy, Cork Harbour (S)	51°50′N, 8°19′W	June 2010	Wild	30	Class C
Ringaskiddy, Cork Harbour (S)	51°50′N, 8°19′W	November 2010	Wild	30	Class C
Garrettstown (E)	51°38′N 8°34′W	June 2008	Wild	30	Class A
Flaxfort Strand (E)	51°38′N, 8°41′W	May 2010	Wild	14	Class B
Lough Hyne (S)	51°30′N, 9°18′W	May 2010	Wild	30	Class A
West coast					
Bantry Bay (SE)	51°40′N, 9°27′W	November 2008	Cultured	15	Class A
Gourma Island (E)	53°13′N, 09°10′W	December 2008	Wild	14	Class A
New Quay (S)	53°14′N, 09°03 W	December 2008	Cultured	14	Class A
Clew Bay (SE)	53°50′N, 09°49′W	December 2010	Wild	30	Class B
Sligo Bay (E)	54°16′N, 8°38′W	February 2011	Cultured	30	Class A
Sligo Bay (E)	54°16′N, 8°38′W	June 2011	Cultured	30	Class A
North coast					
Mulroy Bay (S)	55°15′N, 07°46′W	January 2009	Cultured	15	Class A
Ballyhiernan (E)	55°15′N, 07°42′W	December 2008	Wild	15	Class A
Lough Foyle (S)	55°7′N, 07°5′W	October 2010	Wild	19	Class A
East coast					
Carlingford Lough (S)	54°7′N, 06°19′W	July 2010	Cultured	60	Class A
Silver Strand (S)	2°57′N, 6°00′W	July 2011	Wild	30	Class A
Poulshone Strand (S)	52°37′N, 6°13′W	July 2011	Wild	30	Class A
Wales, UK					
The Menai Strait (S)	53°08′N, 4°19′W	May 2011	Cultured	30	Class B
Gallows Point (S)	53°15′N, 4°06′W	May 2011	Wild	30	Class B
Swansea Bay (SE)	51°36′N, 3°54′W	June 2011	Wild	30	Class B

E, exposed; SE, semi-exposed; S, sheltered.

of the primer Me15 (5′-CCAGTATACAAACCTGTGAAGA-3′), 0.5 μl of the primer Me16 (5′-TGTTGTCTTAATGGTTTGTAAGA-3′), 1 μl of Taq polymerases and 1 μl of total DNA under the following conditions: 94 °C for 30 s, 55 °C for 45 s and 70 °C for 90 s (40 cycles). Electrophoresis of the amplification products was conducted in a 2% agarose gel. The expected product size for *M. galloprovincialis* was 126 bp, for *M. edulis* 180 bp and both bands occurred simultaneously at 126 bp and 180 bp in hybrids.

2.4. PCR screening for haplosporidian infection

Mussel DNA (n = 455) was also screened with generic haplosporidian HAP-F1 and HAP-R3 primers (Renault et al., 2000) and the ssu980 and HAP-R1 primers (Renault et al., 2000; Molloy et al., 2012).

Eight replicate PCR products from an individual mussel were purified using the Qiaquick gel extraction kit (Qiagen) prior to direct sequencing. Both the forward and reverse strands of DNA for both samples were sequenced commercially (MWG eurofins). Each sequence was matched against the National Centre for Biotechnology Information (NCBI) nucleotide database with BLASTn (Basic Local Alignment Search Tool) to identify the species present. The obtained sequences were imported into Mega 5.1 (Tamura et al., 2011) and aligned using the ClustalW algorithm (using default settings). The aligned sequences were analysed in PAUP*4.0b10 (Swofford, 2002).

2.5. Statistical analysis

In this study, a reliable statistical comparison of disease prevalence and diversity between species and hybrids was not possible due to the numbers of each parent species and their hybrids sampled being rarely equally distributed. In a previous study, a similar finding was observed and it was concluded in that study that statistically significant numbers of all mussel species are necessary to accurately assess the effect of species on health parameters (Bignell et al., 2008).

Significant difference in the number of sites infected with microparasites or macroparasites was calculated between (a) wild and cultured mussel stocks, (b) sites with varying water quality and (c) sites with varying exposures using chi-square (χ^2) analysis with significance determined at $p < 0.05$.

3. Results

In Ireland, mytilid samples were collected from thirteen Class A sites, five Class B sites and three Class C sites. In Wales, *M. edulis* were collected from two Class A sites and one Class B site. Exposure at each site was also evaluated and classified as sheltered, semi exposed or exposed (Table 1).

3.1. Screening of individual mussels to identify to species level

Of the 365/841 Irish mussels screened by PCR, 83.8% (306) were *M. edulis*, 8.8% (32) were hybrids and 7.4% (27) were *M. galloprovincialis*. *M. edulis* was the only species detected at the three Welsh sites (n = 90) (Table 2).

3.2. Screening to assess health status

No significant parasite infections i.e. either parasites that cause significant mortalities or very heavy infestations were observed at the Irish sites during the study. The most common organisms observed were: ciliates, trematodes, prokaryote inclusion bodies

Table 2
PCR analysis of mussel samples from eleven Irish and three Welsh sites to determine relative abundance of *Mytilus edulis*, *Mytilus galloprovincialis* and hybrids.

Sample site	Sample date	*M. edulis*	*M. galloprovincialis*	Hybrid
Irish sites				
South coast				
Roches Point	June 2010	63% (12/19)	16% (3/19)	21%(4/19)
Whitegate	June 2010	68% (13/19)	0	32% (6/19)
Whitepoint	June 2010	94% (17/18)	0	6% (1/18)
Ringaskiddy	June 2010	80% (16/20)	5% (1/20)	15% (3/20)
	November 2010	77% (23/30)	7% (2/30)	17% (5/30)
West coast				
Clew Bay	December 2010	53% (16/30)	23% (7/30)	23% (7/30)
Sligo Bay	July 2011	86% (52/60)	7% (4/60)	7% (4/60)
North coast				
Lough Foyle	October 2010	95% (18/19)	0	5% (1/19)
East coast				
Carlingford Lough	July 2010	100% (60/60)	0	0
Silver Strand	July 2011	100% (30/30)	0	0
Poulshone Strand	July 2011	100% (30/30)	0	0
Dunmore East	February 2011	64% (19/30)	33% (10/30)	3% (1/30)
Welsh sites				
Menai Strait	May 2011	100% (30/30)	0	0
Gallows Point	May 2011	100% (30/30)	0	0
Swansea Bay	June 2011	100% (30/30)	0	0

(round to oval shaped, intractyoplasmic, dark staining structures 5–10 μm in size found in a range of tissues including the gills, digestive diverticulum and gonad), *Nematopsis* spp., the copepod *Mytilicola intenstinalis*, turbellaria and unidentified bacterial infections. The pea crab, *Pinnotheres pisum*, was noted in several mussels at Cobh, Cork Harbour (Figs. 2 and 3). All of the observed organisms were detected in *M. edulis* exclusively and the only parasite group observed in *M. galloprovincialis* and hybrids (59/365 mussels) were trematodes (16.2%).

Ciliates (0–23% prevalence of infection) were observed on mussel gills at 11/18 sites with mussels in Cobh having the highest prevalence and intensity of infection. *Nematopsis* spp. oocysts within the mantle and connective tissue were present in *M. edulis* at Ballyhiernan (80% prevalence of infection) and Garretstown (40% prevalence of infection) with fewer than ten oocysts being visualised in each mussel on the northern and southern coasts.

Trematodes (sporocyst but mainly metacercaria stages) were observed in *M. edulis*, *M. galloprovincialis* and hybrids. In Ireland, Dunmore East (30%) had the highest prevalence of infection and in Wales, mussels at Gallows Point had 40% prevalence of infection with metacercaria. The copepod *M. intestinalis* was detected at 11/18 sites in Ireland in *M. edulis* exclusively and was observed in the connective tissue and digestive tubules with the highest prevalence of infection being observed in Sligo Bay (23%). Similarly 27% of mussels at two of the three Welsh sites were infected with *M. intestinalis*. An unidentified turbellarian was detected in *M. edulis* in Lough Foyle (8% prevalence of infection) on the northern coast and pea crabs were detected in *M. edulis* in Cobh (3% prevalence) on the southern coast.

At two sites, Ringaskiddy and Sligo Bay, two samples were taken in different seasons with results indicating that seasonal variation in the parasite diversity present and their prevalences occurred. In the Ringaskiddy June 2010 sample, three parasites were observed; ciliates (13%), trematode spp. (13%) and *M. intestinalis* (7%). In the subsequent Ringaskiddy sample taken in November 2010, trematode spp. (57%) were detected at a much higher level but there was no evidence of ciliates or copepods. In Sligo Bay in February 2011, ciliate spp. (8%), *M. intestinalis* (18%) and trematode spp. (13%) were detected and in June 2011 variation in the prevalence of all three had occurred at 4%, 28% and 7%, respectively.

In Wales, mussels screened by PCR using the generic haplosporidian primers amplified a product (350 bp) in 18/30 mussels (60% prevalence) from the Menai Strait but direct sequencing was unsuccessful. Using primers (ssu980 and HAP-R1), a product was amplified at 430 bp in 1/30 mussels, a product had also been amplified in this individual using the HAP-F1 and HAP-R3 primers. Blastn analysis of the DNA sequences (two forward and two reverse) isolated from the Welsh *M. edulis*, confirmed the DNA to be that from a previously undescribed Haplosporidian sp. (Accession no. KC852876 and KC852877) most similar to *Minchinia chitonis* (Accession no. AY449711.1, 95–100% Query coverage, 92–93% Maximum identity) and a haplosporidian of the Florida marsh clam *Cyrenoida floridana* (Accession no. AY449712.1, 90–98% Query coverage, 93–95% Maximum identity). A phylogenetic tree was generated based on the majority rule jackknife consensus parsimony analysis with 1000 pseudo replicates with 75% character deletion using a heuristic search with 1000 random sequence additions each (Fig. 4). While unidentified organisms were observed in the histology of those corresponding mussels, these organisms could not be definitively identified as being haplosporidian. Additionally, tissue was unavailable for *in situ* hybridization (ISH) to be carried out to confirm an actual infection.

Overall there was no difference between wild and cultured mussels, however, two sites with cultured mussels in Ireland had no evidence of parasites or pathologies. No significant difference, in the presence or the absence of microparasites ($P = 0.225$) and macroparasites ($P = 0.059$), was observed beween wild and cultured mussels.

3.3. Effect of water quality and site exposure

In Ireland, low levels of bacterial infections associated with eroded gills and poor water quality were only observed in *M. edulis* at two sites within Cork Harbour, Cobh (17% prevalence) and Whitegate (3% prevalence). High intensity prokaryote inclusions were detected in *M. edulis* at Woodstown Beach (60%) on the south east coast and were found in a range of tissues including the gills, digestive diverticulum and gonad (Fig. 2).

In Ireland, parasites were detected at all sites except two sheltered Class A water quality sites, New Quay on the west coast and Mulroy Bay on the north coast. The most diverse group of parasites was observed at Cobh, a Class C sheltered site, with four groups of parasites being detected with gill damage also in these mussels. No clear association with water quality and

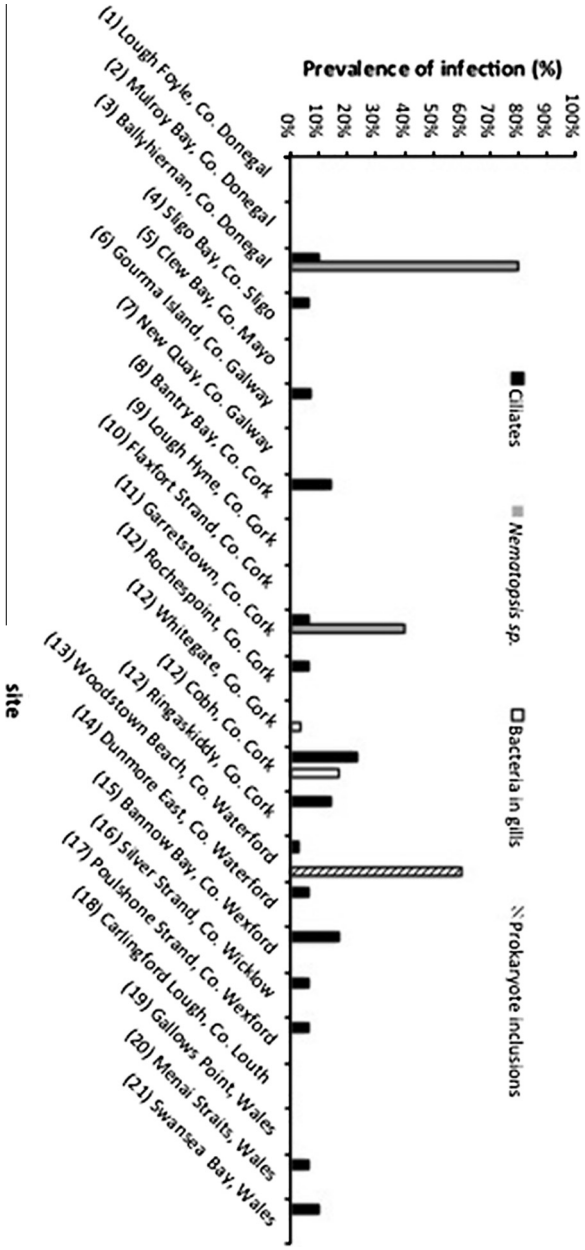

Fig. 2. Prevalence of infection (%) of microparasites in *Mytilus* spp. at Irish and Welsh sites. Number in brackets denotes site number.

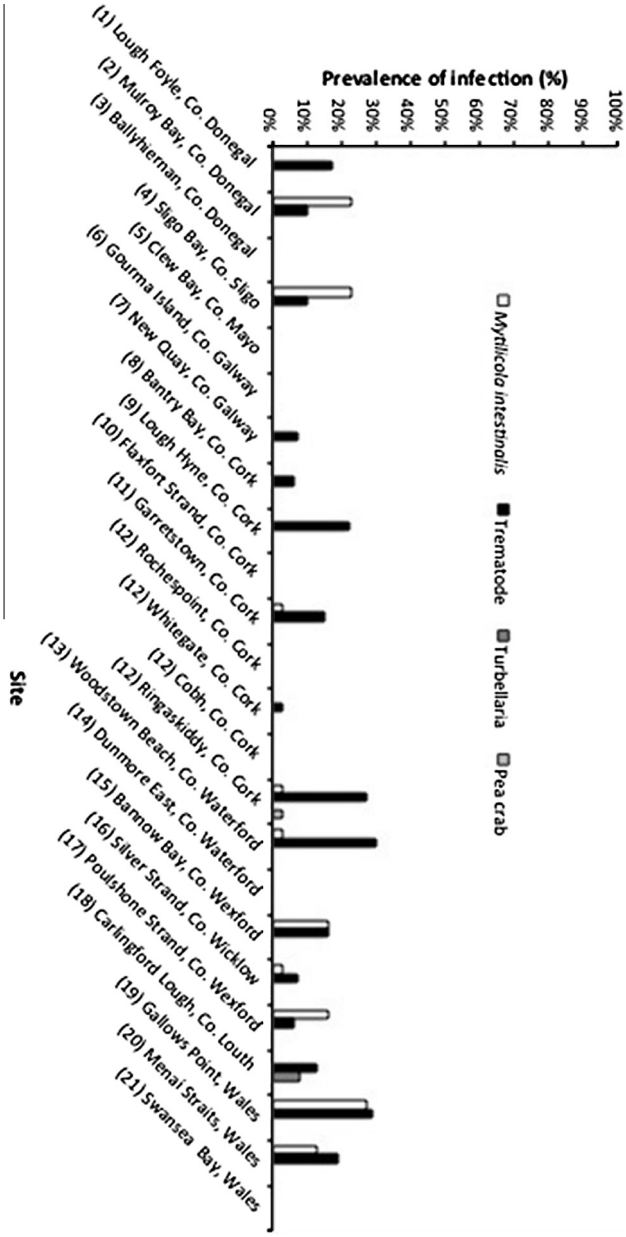

Fig. 3. Prevalence of macroparasites in *Mytilus* spp. at Irish and Welsh sites.

parasites was observed at the remaining Irish sites. In Wales, parasites were detected at all three sites which all had Class B water quality.

The number of parasite groups was greater at sheltered sites ($n = 12$) compared to exposed ($n = 8$) and semi-exposed ($n = 4$) sites. No significant difference was observed, in the detection of microparasites, when all sites of varying water quality were compared, however, a significant difference ($P = 026$), in the detection of macroparasites, was observed between Class A and Class B rated sites, with more macroparasites being observed at Class A sites. No significant difference in the detection of microparasites was observed between the three types of site exposure, however, a significant difference ($P = 0.009$) was observed in the detection of macroparasites between exposed and sheltered sites, with more macroparasites being observed at sheltered sites.

4. Discussion

M. edulis was the dominant species found around the coast of Ireland and was the only mussel species found in the Irish Sea. A diverse community of both micro- and macro-parasites were observed, however, no significant pathogens, *Marteilia refringens* for example, were detected and few of the parasites present were at intensities that would cause significant pathologies or mortality. However, of significance is the detection and molecular confirmation of a previously undescribed haplosproidian-like organism (HLO) in *M. edulis* at a single Welsh site. The impact of this haplosporidian on that mussel stock is yet to be determined. Bignell et al. (2008) describe a haplosporidian infection in *Mytilus* spp. from the Exe estuary and Southhampton Water, UK, in 2004 and 2005. Multinucleated plasmodia were observed but molecular analysis was not carried out in that study, so it could not be confirmed if they are the same species or not.

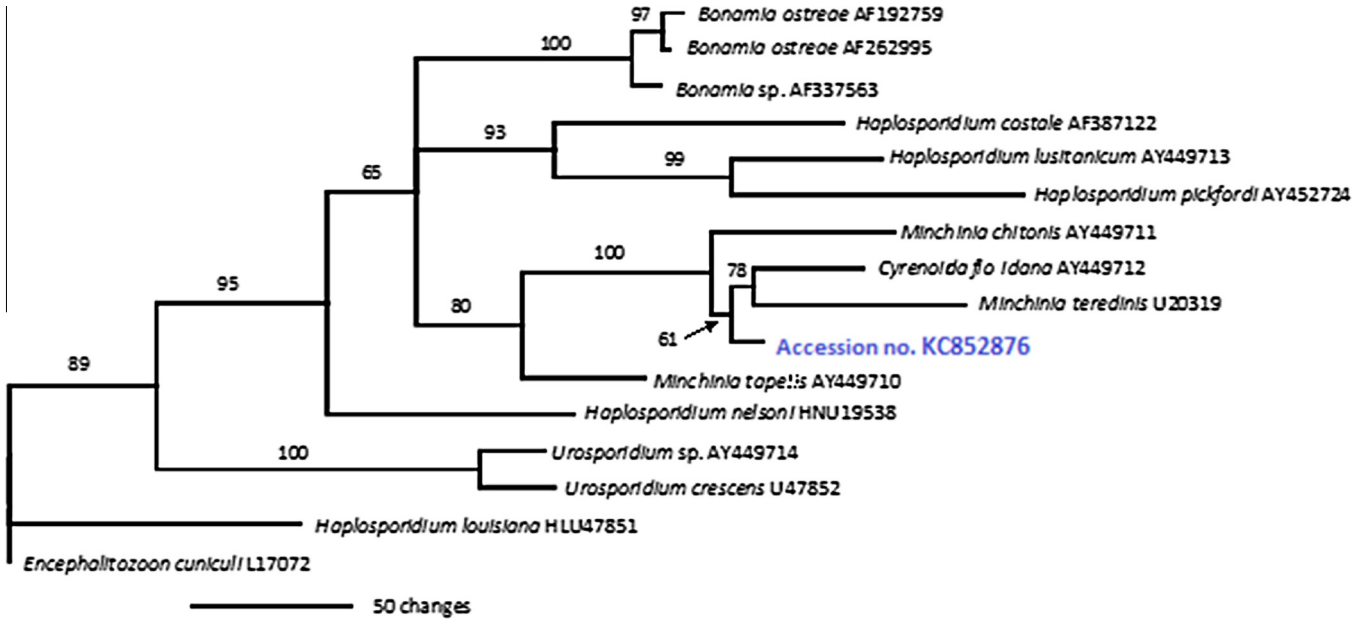

Fig. 4. Majority rule jackknife consensus resulting from parsimony analysis with the SSU rDNA gene. Each sequence name is followed by the GenBank accession number. The haplosporidian detected in this study is labelled Accession no. KC852876.

Few of the parasites observed in this study cause major mortalities in *Mytilus* spp. though under stressful conditions in other bivalves they can multiply and cause mortalities (Otto et al., 1979; Figueras et al., 1991; Villalba et al., 1997). For example, ciliates are ubiquitous in mytilid spp. (Cremonte et al., 2005) but can feed on the cells and tissues of certain molluscs while living and reproducing in their tissues (Elston et al., 1999) and intensity of ciliate spp. that occur in fish gills have been shown to increase with industrial effluent (Overstreet and Howse, 1977). *Nematopsis* spp. have been associated with mass mortalities of cockles and clams in Portugal with 82% prevalence of infection in *Cerastoderema edule* (Azevedo and Cachola, 1992) and in *M. galloprovincialis* (100% prevalence of infection), *C. edule* and the lagoon cockle, *Cardium glaucum,* in Galicia, Spain (Sprague and Orr, 1955; Estevez et al., 1998) while prokaryote inclusions have been associated with mass mortalities in the deep sea scallop *Placopecten magellanicus* in North America (Gulka et al., 1983). Heavy trematode sporocyst infections can cause a decrease in gametogenesis, castration and sometimes death (Calvo-Ugarteburu and McQuaid, 1998).

All parasite groups were found in *M. edulis* while trematodes were only detected in *M. galloprovincialis* and hybrids. Fuentes et al. (2002) noted that mytilid hybrids are more susceptible to being parasitized than the parental species, however, that was not observed in this study. This finding may reflect parasite–host species specificity, a "dilution effect" due to the much lower density of *M. galloprovincialis* and hybrids present compared to *M. edulis* at each site or the possibility that *M. galloprovincialis* and hybrids infected with parasites are unable to establish themselves.

Parasite diversity and prevalence increased with decreasing wave exposure and were more prevalent in wild mussel populations. In this study, water quality did not influence the diversity and abundance of parasites present. An association of parasite diversity and abundance with poor water quality has been observed in previous studies (Lowe and Moore, 1979; Auffret, 1988; Elston et al., 1999; Calvo-Ugarteburu and McQuaid, 1998; Svärdh, 1999; Svärdh and Johannesson, 2002; Bignell et al., 2008). Morley et al. (2004) suggested that mussels exposed to pollutants and heavy metals can be more susceptible to secondary trematode infection, however, mussel stocks infected with trematodes in this study were found in both polluted and nonpolluted sites and were located on the upper to mid shore, which would increase their contact with other potential hosts of this parasite group.

It can be concluded from the results of this study that parasites currently detected in Irish and Welsh mytilid populations, with the exception of the previously undescribed haplosporidian-like organism, do not pose a significant risk to either wild or cultured populations. Although *M. edulis* were infected with a greater diversity and prevalence of parasites compared to *M. galloprovincialis* and hybrids, it was also the dominant species present at each sample site. This would indicate that parasite infection is not having a negative effect on the population size of *M. edulis*. Wild mytilid stocks had greater parasite diversity and prevalence compared to the cultured stocks, however, this difference was not significant and certain parasites may not be present in cultured mussels, such as trematode spp., as other hosts which play a role in their life cycle may be absent due to the culture method or environment. Potential future changes in these coastal communities such as climate change and associated stressors may further impact on host:parasite dynamics and these relationships need to be evaluated further particularly in relation to the haplosporidian observed in the current study whose future impact is unclear.

Acknowledgments

The authors would like to thank the shellfish growers who contributed material for the study. The project was part funded by the European Regional Development Fund (ERDF) through the Ireland Wales programme (INTERREG 4A) SUSFISH. Dr. J. Finarelli is thanked for help with constructing the phylogenetic tree.

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