Characterisation of *Marteilia cocosarum* in the Wash Estuary, UK, linked to mass mortalities of cockles (*Cerastoderma edule*), and its relationship to closely related species

Running page head: *Marteilia cocosarum* in The Wash

Anna Tidy*[1], Ron Jessop[2], Georgia M. Ward[1], Matthew J. Green[1], Kelly S. Bateman[1,3], David Bass[1,3,4,] Jasmine E. Hunt[1,5], Stuart H. Ross[1], Chantelle Hooper[1,3]

[1]Centre for the Environment, Fisheries and Aquaculture Science (Cefas), Weymouth Laboratory, Weymouth, Dorset, DT4 8UB, United Kingdom

[2]Eastern Inshore Fisheries and Conservation Authority (EIFCA), North Lynn Business Village, Bergen Way, North Lynn Industrial Estate, King's Lynn, Norfolk, PE30 2JG, United Kingdom

[3]Sustainable Aquaculture Futures, Biosciences, College of Life and Environmental Sciences, University of Exeter, Exeter, United Kingdom

[4]Department of Life Sciences, Natural History Museum, London, SW7 5 BD, United Kingdom

[5]School of Biosciences and Medicine, University of Surrey, Guildford, Surrey, GU2 7XH, United Kingdom

* Corresponding author: anna.tidy@cefas.gov.uk

Abstract

Globally, *Marteilia* parasites have been associated with significant mass mortality events in populations of commercially important bivalve molluscs, frequently resulting in large-scale fishery collapses and substantial socio-economic impacts. The Wash Estuary, UK, supports several bivalve fisheries, and among these, common cockles *Cerastoderma edule* have suffered unusually high mortalities since 2008. We investigate potential causes of these mortalities, and confirm infection with *Marteilia cocosarum*, strongly associated with cockle moribundity, also confirming its presence in archived samples collected in 2009. Molecular and light microscopy screening of samples collected during mortality events in 2021, including healthy (buried) and moribund (weak, unable to bury) cockles, indicated high prevalence of *M. cocosarum* in moribund cockles (PCR incidence up to 95%) in contrast to healthy cockles (up to 42%), suggesting an association between cockle moribundity and *Marteilia* infection. Analysis of the full ribosomal RNA array identified consistently different nucleotides between *M. cocosarum* infections in The Wash (denoted as genotype WE) and those in Wales (denoted genotype BI). 83% of infections in The Wash could be identified as *M. cocosarum* WE and 12% as *M. cocosarum* BI, with both genotypes recovered from 5% of infected animals. Histopathologically, *M. cocosarum* WE infects the gill, mantle and connective tissues, identical to observations of *M. cocosarum* infecting Welsh cockles. Ongoing cockle mortalities in The Wash raise concerns regarding the sustainability of this resource ecologically and economically. Additional measures may be required to reduce the spread of this pathogen, noting that its distribution beyond The Wash and Wales is currently unknown.

Key words: Cockle, *Marteilia,* pathogen, fishery, Ascetosporea, aquatic disease

1. Introduction

Marteilia (Ascetosporea, Paramyxida) species are of worldwide significance owing to their detrimental impacts on bivalve mollusc hosts, particularly those of economic importance. Nine species are currently assigned to the genus (Skujina et al., 2022), affecting hosts including oysters (*Ostrea, Crassostrea* and *Saccostrea* spp.), mussels (*Mytilus, Modiulus* and *Xenostrobus*), cockles (*Cerastoderma* spp.) and clams (*Ruditapes, Scrobicularia, Solen* spp.), as most recently reviewed by Ward et al., (2016). The mode of infection and pathogenicity is known to vary depending on the host, with most species infecting the digestive gland (Berthe et al., 2004), and several *Marteilia* species are notorious for their deleterious effect on cultivated bivalve hosts.

In European populations of the common cockle *Cerastoderma edule*, *Marteilia* parasites have previously caused mass mortalities in two Spanish regions, and were linked to mortalities in Wales, United Kingdom. The cockle fisheries of the Ría de Arousa, Galicia, Spain, were previously amongst the most productive shellfisheries in northwestern Spain, but have suffered tremendous collapses since 2012, linked to infection with a *Marteilia* species, initially designated *Marteilia* “type C”, and later formally described as *M. cochillia* (Carrasco et al., 2012, 2013; Villalba et al., 2014). Following recurrent mortalities in several key Welsh cockle populations since 2002 (Murray & Tarrant, 2015; Woolmer, 2013; Burdon et al., 2014), Skujina et al. (2022) associated these mortalities with infection with a novel *Marteilia* species, *M. cocosarum*, a close but phylogenetically and phenotypically distinct sister species of *M. cochillia*. Unlike most other characterised *Marteilia* species, *M. cocosarum* was shown to primarily infect the base of the gill and mantle tissue, with parasite stages distributed through the connective tissues. This unique tissue tropism distinguished infection with *M. cocosarum* from other species, including *M. cochillia* (Skujina et al. 2022).

Investigation of the distribution of *M. cocosarum* in *C. edule* populations around British and Irish coasts using molecular screening only found evidence of its presence around the Welsh coastline (Skujina et al. 2022). *M. cocosarum* was found at high prevalence (48-79%) in cockles across the Welsh sampling sites and was tentatively implicated in cockle mortalities at these locations, in conjunction with additional stressors. However, a lack of data prohibited more conclusive links between parasite infection and mortality events being drawn.

Cockles are known to be infected with many other eukaryotic parasites including metazoans (trematode and nematode worms; copepod and decapod crustaceans), and microbial eukaryotes (including Microsporidia, Alveolata, and other Ascetosporea), as reviewed in de Montaudouin et al., (2021). Despite the large number of eukaryotic parasites known to infect cockles, their prevalence in populations is typically low, and detrimental effects due their

presence are unlikely (de Montaudouin et al., 2021). Bacterial and viral agents known to infect cockles are relatively few compared to eukaryotic organisms. The only bacterial species reported to be a threat to cockle production is *Vibrio aestuarianus* (also responsible for mortalities of other bivalve species) which has been identified as the causative agent of cockle summer mortality events in France (Garcia et al., 2021). A retrovirus was initially linked to the presence of disseminated neoplasia (DN) in multiple bivalve species (Romalde et al., 2006), however this has never been confirmed. Despite the causative agents and transmission of DN being currently unknown, DN in cockles has been linked to mass mortalities in Spain (Villalba et al. 2001; Díaz et al. 2016)

The Wash estuary, located on the border of Norfolk and Lincolnshire, United Kingdom, is the largest nature reserve in the UK (Natural England., 2010). Not only is it a hugely important wetland site for wild mammal and bird populations, it also supports numerous molluscan fisheries including *C. edule*, blue mussel *Mytilus edulis* and, to a lesser extent, whelks *Buccinum undatum* (Eastern IFCA., 1992; 2021). Since 2008, cockle stocks in The Wash have suffered unusually high mortalities, particularly affecting larger cockles (>14mm width), leaving beds dominated by smaller juvenile stocks (Eastern IFCA., 2019). These mortalities are considered atypical by the Eastern Inshore Fisheries Conservation Authority (EIFCA) who conduct annual spring surveys and manage the fishery, in that they do not resemble die-offs which routinely occur due to natural events or normal annual variation. Cockle populations have been reported to periodically undergo natural mortality events, including “ridging out” within dense beds, where the larger, less active cockles are forced out of the sediment by younger, more active cockles (Eastern IFCA., 2019). The widespread, atypical losses in Wash cockle populations commonly occur in summer months, during which moribund individuals can be observed gaping on the surface of sandbanks, with slow response to stimuli and overall weakness (Eastern IFCA., 2011). It has also been reported that faster-growing beds appear to be particularly vulnerable to these mortalities (Eastern IFCA., 2011). In fast-growth beds, the cockles reach maturity within 1-2 years, whilst slower-growing cockle beds only produce adults of spawning size after 3-4 years. Atypical die-offs occur in cockles according to size class as opposed to age class, and so those that have reached a marketable size in 2 years on some beds are as vulnerable to mortality as those of 4 years on slower-growing beds. On some fast-growth beds, mortality rates have been observed to exceed 90% of produced biomass once cohorts reach a vulnerable size (Eastern IFCA., 2011). Furthermore, since the events predominantly affect larger size-class cockles, irrespective of age, there has been an overall reduction in the mean cockle size within The Wash, and a shift towards smaller cockles being targeted for harvest (Eastern IFCA., 2022). This could result in the unsustainability of the fishery and reduced longevity of stocks into the future if cockles are harvested before

spawning. Moreover, by thinning the stocks at smaller sizes, densities of larger cockles are reduced the following year, perpetuating population reduction. Cockles also hold significant ecological value, particularly through food provision for wild bird populations such as oystercatcher *Haematopus ostralegus* and knot *Calidris canutus* (Stillman et al., 2010), and so widespread population declines also raise the alarm for ecological stability in affected areas.

Cockle mortalities with a similar aetiology have been reported in the Burry Inlet, Wales, UK, since 2004, presenting similar issues for the fishery, and suggesting potential similarities in the causative factor(s) behind these declines (EIFCA, 2011). A comprehensive study of cockle populations in the Burry Inlet was carried out by the Welsh Environment Agency and partners, published by Elliott et al., (2012), which found no direct correlation between the measured water quality parameters and cockle mortality; nor could mortality be conclusively linked to parasite infection as a primary cause, especially as parasite load was found to be higher in populations sampled in the Dee estuary, where mortality incidence was significantly lower. It was concluded that high cockle density, combined with a high energy expenditure and high reproductive output was likely behind population declines, and that more or one other factors (including sediment composition, benthic community composition, water quality or parasite infection) may be contributing to reduced tolerance in cockles already under stress (Elliott et al. 2012). The presence of large foci of haemocyte infiltration was tentatively linked to the presence of haplosporidian parasites (Ascetosporea, Haplosporida), including *Minchinia mercenariae* and *M. tapetis*, though insufficient data were available to confirm this (Longshaw & Malham., 2013). Subsequent studies in Ireland and Spain have confirmed infection of *C. edule* with both species, though infection has not been linked to population declines or mortalities (Albuixech-Martí et al., 2020; Ramilo et al., 2018).

In this study, we 1) Investigate the presence and prevalence of pathogens and pathology potentially associated with cockle mortalities in The Wash by molecular and histopathological screening. 2) Following the detection of *M. cocosarum,* we determine the phylogenetic position of this parasite by sequencing the full ribosomal RNA (rRNA) array. 3) Determine, by revisiting archival cockle specimens from The Wash collected during mortality events in 2009 and 2010, whether *M. cocosarum* was implicated in early cockle mortalities in The Wash, and 4) investigate the presence of other pathogen groups and pathologies previously linked to cockle mortalities, including haplosporidians (*Haplosporidium* and *Minchinia* spp.) and disseminated neoplasia.

2. Materials and methods

2.1 Sample collection

Samples of approximately 50 moribund *Cerastoderma edule* from the surface of the sandbank and 50 buried "healthy" *C. edule* (not showing clinical disease signs) were collected by the Eastern IFCA from three sites in the Wash Estuary between April and July 2021, and in May 2022. Moribund cockles were identified by weakness, gaping, and the apparent inability to bury in the sediment, while healthy cockles were buried in the sediment and showed normal responses to artificial stimuli (i.e. the two valves of the shell could not be prised apart). Animals were transported overnight in coolboxes to the Cefas Weymouth laboratory for processing. A cross-section comprising representative tissues of all organs was fixed in Davidson's Seawater Fixative for histopathology and *in situ* hybridisation, and an equivalent section was fixed in RNALater (Sigma Aldrich, USA) for molecular analyses. Additional sections of mantle and digestive gland tissues (approx. 2 mm^3) were preserved in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer for transmission electron microscopy (TEM). Archived samples were revisited to determine if the pathogens and pathologies identified in this study were present at the start of the annual mortalities occurring. These samples were collected for histopathology only, as above. All samples included in this study, including sampling date and location, and sample size, are shown in Table 1.

Table 1. Summary of samples collected at study sites within The Wash estuary, including site location, sampling date and number of individuals collected. Moribund cockles were collected from the surface sediment, and displayed weakness, gaping and an inability to bury, whereas healthy cockles were dug out of the sediment and showed no clinical signs of reduced health status.

Site name	Site location	Sampling date	Sample codes	Moribund cockles	Healthy cockles	Total
Mare Tail	52° 54' .950" N, 00° 07' .200" E	28/04/2021	RA21014 (1-49, 90-139)	49	50	**99**
		16/05/2022	MT2022	48	0	**48**
Dill's Sand	52° 56' 150" N, 00° 07' 050" E	28/04/2021	RA21014 (50-89, 140-189)	40	50	**90**
		16/05/2022	DS2022	48	0	**48**
Inner West Mark Knock	52° 50' 300" N, 00° 14' 250" E	26/07/2021	RA21022	50	50	**100**
Bob Halls Sand	52° 58' 57.6" N 0° 50' 53.88" E	06/05/2009	PM19194	0	13	**13**

176 *2.2 DNA extraction from RNAlater-fixed tissues*

177 RNALater-fixed cross sections were further dissected to prepare 50 – 100 mg comprising
178 representative tissue from the mantle, digestive gland, gill and adductor muscle. Tissue was
179 homogenised in 800 µl Lifton's buffer (100 mM EDTA, 25 mM Tris-HCl, 1% SDS, pH 7.5;
180 Winnepenninckx et al (1993)) in a 2 ml Lysing Matrix A homogenisation tube (MP
181 Biomedicals,). Homogenisation was performed in a FastPrep-24 (MP Biomedicals) at 5 m/s
182 for 60 seconds. 20 µl Proteinase K (20 mg/ml; Sigma Aldrich) was added to each homogenate
183 before incubation at 56°C for at least three hours on a static dry block. Following Proteinase
184 K digestion, DNA was extracted from 100 µl of homogenate using the Maxwell RSC Tissue
185 DNA kit, following the manufacturer's instructions, on a Maxwell 48 Rapid Sample
186 Concentrator (RSC) instrument (both Promega) in batches of 47, with a negative control
187 extraction (i.e. no homogenate added) performed in each batch. Resulting DNA extracts were
188 diluted 1:10 in molecular grade water for use as PCR templates.

189 *2.3 DNA extraction from formalin fixed, paraffin-embedded tissues*

190 Paraffin-embedded blocks from samples collected in May 2009 were imaged and measured
191 to calculate the approximate number of 10 µm sections required to obtain 4 mm^3 tissue. DNA
192 was extracted from sectioned formalin-fixed, paraffin embedded (FFPE) sections using the
193 QIAamp DNA FFPE Advanced UNG kit (Qiagen) using the manufacturer's protocol including
194 the Uracil-N-Glycosylase (UNG) repair step, with the following modifications: Sections were
195 dissolved in a total volume of 500 µl Deparaffinisation Solution, and incubated at 56°C for 3
196 minutes to dissolve paraffin was performed twice, in order to fully remove all paraffin from the
197 sections. DNA was eluted into a volume of 50 µl buffer ATE after incubation on the column for
198 5 minutes, and the eluate reapplied to the spin column and incubation repeated to maximise
199 DNA yield. All optional steps in the manufacturer's protocol were included, and all "vigorous"
200 vortexing steps were performed at 2700 rpm for 10 seconds. DNA was repaired using the
201 NEBNext FFPE DNA Repair module v2 (New England Biolabs), using a modified version of
202 the manufacturer's protocol for user-supplied reagents (Section 3). Enzymatic incubation
203 steps were performed as directed, but cleanup steps using sample purification beads were
204 omitted to reduce loss of DNA.

205 *2.4 PCR screens and sequencing*

206 The sequences of all primers used in this study are shown in Table 2. All reactions were
207 performed in 20 µl volumes, comprising 1× Green GoTaq Flexi Buffer (Promega), 2.5 mM
208 $MgCl_2$ (Promega), 0.4 mM dNTP mix (Meridian Bioscience, USA), 10 µg bovine serum albumin
209 (BSA) (New England Biolabs), 0.5 µM each primer, 0.5 U GoTaq G2 Flexi DNA polymerase
210 (Promega), and 1 µl diluted template DNA. Reaction composition was identical for each round

211 of nested PCR, with 1 µl first round product used as a template for the second round. PCR
212 screens of all *C. edule* DNA extracts were performed using the hemi-nested, paramyxid-
213 targeted 18S ribosomal RNA (rRNA) primer set B (Para1fGW/ParaGenRGW and
214 Para3fGW/ParaGenRGW) and cycling conditions of Ward et al. (2016). A further *Marteilia*-
215 specific nested PCR, targeting the ITS region, was performed to characterise a potentially
216 more phylogenetically informative region of the rRNA array, using the nested primer set
217 (MartDBITSf1/MartDBITSr1 and MartDBITSf2/MartDBITSr2) and cycling conditions of Kerr et
218 al. (2018). Finally, samples collected in 2021 were screened using the nested, haplosporidian-
219 targeted primer set (C5fHap/Sb1n and V5fHapl/Sb2nHap) of Hartikainen et al., (2014), using
220 the modifications presented in Ward et al., (2019). Resultant amplicons were visualised on
221 1.5% (*w/v*) agarose-TBE gels stained with GreenSafe (NYZ Biotech) alongside 5 µl 100 bp
222 molecular marker (100 bp DNA Ladder; Promega). PCR products were purified using AMPure
223 XP sample purification beads (Beckman Coulter), and bidirectionally Sanger sequenced using
224 the second round primers using the Eurofins Genomics PlateSeq service (Eurofins
225 Genomics).

226 For paramyxid amplicons that produced mixed chromatogram traces, amplicons were ligated
227 into pGEM-T Easy Vector Systems (Promega) and transformed into *E. coli* JM109 competent
228 cells (Promega) before plated on LB-Ampicillin agar. Transformed colonies were picked and
229 amplified using M13 primers (Messing., 1983), before visualisation, purification and Sanger
230 sequencing as above, using the M13 plasmid primers.

231 DNA extracts from FFPE material were screened using generic *Marteilia* SSU primers SS2
232 (Le Roux et al. 1999) and SAS1_MOD, based on the original SAS1 primer of Le Roux et al.
233 (1999) but with an additional C base removed to improve complementarity to European
234 *Marteilia* spp. Reactions were conducted in 20 µl volumes using the same composition as
235 above, but using 5 µl repaired DNA as template. Cycling conditions consisted of an initial
236 denaturation at 95°C for 5 minutes, followed by 45 cycles of 95°C for 30 seconds, 55°C for 1
237 minute and 72°C for 1 minute. A final extension step at 72°C for 10 minutes was performed,
238 before reactions stored at 4°C. The complete PCR product (i.e. 20 µl) was visualised on 2%
239 (*w/v*) agarose-TBE gels. Resultant bands were excised using a sterile blade and purified using
240 the Monarch DNA Gel Extraction Kit (New England Biolabs). Purified DNA was eluted into a
241 volume of 20 µl, and unidirectionally Sanger sequenced using the forward primer using the
242 Eurofins Genomics TubeSeq Supreme service (Eurofins Genomics, Germany. Resultant
243 chromatogram traces were trimmed to remove primer sequences and low quality bases using
244 MEGA v11 (Tamura et al. 2021), and resultant sequences aligned against full-length small
245 subunit rRNA sequences for European *Marteilia* spp. in order to confirm identity. Due to their
246 short length, these sequences were not included in further analyses.

247 **Table 2.** Sequences of primers used in this study for targeted PCR screens and sequencing, ribosomal RNA
248 sequence extension, and generation of digoxigenin-labelled oligonucleotide probes for *in situ* hybridisation.

PCR target	Primer name and sequence (5'- 3')	Amplicon length (bp)	Reference
Paramyxid partial 18S (V5 – V7 regions)	Round 1 – Para1fGW: GGGCGAGGGGTAAAATCT ParaGenRGW: GTGTACAAAGGRCAGGGACT Round 2 – Para3fGW: GGCTTYTGGGAGAKTACGGC ParaGenRGW: GTGTACAAAGGRCAGGGACT	420	Ward et al. (2016)
European *Marteilia* spp. ribosomal internal transcribed spacer (ITS) regions	Round 1 – MartDBITSf1: CTCGTGGAGCGGGTCTACCG MartDBITSr1: TATCACGCCGCTGAATGCTTTCG Round 2 – MartDBITSf2: TACGCCGTGCCGAGAGGTAC MartDBITSr2: GCGTCCTTCTACGGCTCACGATC	1,100	Kerr et al. (2018)
Haplosporidian partial 18S (V5 – V9 regions)	Round 1 – C5fHap: GTAGTCCCARCYATAAACBATGTC Sb1n: GATCCHTCYGCAGGTTCACCTACG Round 2 – V5fHapl: GGACTCRGGGGGAAGTATGCT Sb2nHap: CCTTGTTACGACTTBTYCTTCCTC	650	Hartikainen et al. (2014)
SMART2 *Marteilia*-generic ISH probe 18S	SS2: CCGGTGCCAGGTATATCTCG SAS1_MOD: TTCGGTGGTCTTGAAAGGC	230	Le Roux et al. (1999) ; This study
Marteilia first internal transcribed spacer (ITS1) to 28S	Mcoch-F: CTCTGTCCGGTCAAAGCCTA LSU8799r_degen: CGAAGRATCAAAAAGCRVCGTC	4,495	Villalba et al. (2014) Hooper et al. (2023)
Marteilia 28S – IGS-18S-ITS1	Mcoch-R: GTGACGYGCAYGARTGGADYAAC CH7939-7962: AATTCGCAGCCCACAAAG	*c.* 6,600	Villalba et al. (2014) Hooper et al. (2023)
European *Marteilia* complete 18S	EuroMarteilia30F : CTAGCGTTCGAGACTAAGCC ParaGenRGW : GTGTACAAAGGRCAGGGACT	1,605	Skujina et al. (2022)

249

250 *2.5* Marteilia *ribosomal RNA array amplification and sequencing*

251 To generate full rRNA arrays for the *Marteilia* lineage detected using group-targeted PCRs,
252 long-range PCR was carried out on PCR-positive individuals (n = 14). Amplicons covering the
253 first internal transcribed spacer region (ITS1), 5.8S gene, second internal transcribed spacer
254 region (ITS2) and partial large subunit 28S gene (hereafter referred to as ITS1-5.8S-ITS2-
255 28S) were amplified using primer Mcoch-F (Villalba et al. 2014) and LSU8799degen (Hooper
256 et al. 2023). Amplicons comprising the 28S gene, intergenic spacer region (IGS), external
257 transcribed spacer (ETS), complete 18S and ITS1 (hereafter 28S-IGS-ETS-18S-ITS1) were
258 amplified using primers Mcoch-R (Villalba et al. 2014) and CH7939-7962 (Hooper et al. 2023).
259 Cycling conditions for both amplicons were as described in Hooper et al. (2023).

260 Both ITS1-5.8S-ITS2-28S and 28S-IGS-18S-ITS2 amplicons were prepared for Illumina
261 sequencing using the Nextera XT DNA Library Preparation Kit (Illumina) following the
262 manufacturer's protocol but using half reaction volumes. Libraries were sequenced on a
263 MiSeq Nano reagent kit (500 cycles) on an Illumina MiSeq platform (Illumina). 28S-IGS-18S-
264 ITS1 amplicons were prepared for Nanopore sequencing using the PCR Barcoding Kit (Oxford
265 Nanopore Technologies, UK) following the kit protocol, and sequenced on Flongle flow cells
266 on a MinION Mk1C platform (both Oxford Nanopore Technologies).

267 Based on the analysis of the full rRNA array sequences, a further PCR covering a region of
268 the array in the 18S gene which discriminated between *Marteilia* identified in The Wash from
269 other *Marteilia* species was performed to demonstrate that differences seen in the full array
270 were consistent across all available samples. The full 18S sequence was amplified using
271 primers EuroMarteilia30F and ParaGenGW, and the cycling conditions presented in Skujina
272 et al. (2022).

273 *2.6 Analysis of ribosomal RNA array sequences*

274 Bidirectional Sanger sequence traces generated from 18S paramyxid-targeted and ITS1
275 *Marteilia*-targeted specific PCRs were trimmed to remove primer sequences and low quality
276 basecalls using MEGA v11 (Tamura et al., 2021), before reads were concatenated and then
277 aligned against existing sequence data for *M. cocosarum* and *M. cochillia* using MAFFT v7.0
278 (L-ins-i algorithm) (Katoh & Standley., 2013). The resulting alignment was viewed in AliView
279 v1.26 (Larsson., 2014). Indels and base changes in the newly-generated sequences
280 comparative to the two closely-related *Marteilia* species were observed and counted by eye.

281 Analysis of long-range amplicon data generated using Illumina and Nanopore platforms
282 followed that used by Hooper et al. (2023) for the analysis of *Marteilia* and *Paramarteilia*
283 sequence data.

284 *2.7 Phylogenetic analysis*

285 A Bayesian consensus tree was constructed based on all available paramyxid 18S sequence
286 diversity to determine the phylogenetic placement of the *Marteilia* species detected in cockles
287 in The Wash, relative to other paramyxid lineages. A second Bayesian consensus tree, using
288 full transcribed rRNA alignments (ETS-18S-ITS1-5.8S-ITS2-28S) generated in this study
289 (alongside publicly available rRNA arrays for *M. cocosarum, M. cochillia and M. octospora*)
290 was then constructed. Both phylogenies were constructed using MrBayes v3.2.7 (Ronquist et
291 al., 2012) on the CIPRES Science Gateway (Miller et al., 2010). Analysis used two separate
292 MC3 runs, carried out for two million generations with one cold and three hot chains. The first
293 500,000 generation were discarded as burn-in, and trees sampled every 1000 generations.
294 Resultant consensus tree files were viewed using FigTree v1.4.4 (Drummond & Rambaut.,
295 2007).

296 *2.8 Histopathology and Transmission Electron Microscopy (TEM)*

297 Sections of cockle tissue including the adductor muscle, gill and digestive gland were fixed in
298 Davidson's Seawater Fixative for 24 hours, before transfer to 70% industrial denatured alcohol
299 (IDA) (Pioneer Research Chemicals Limited). Fixed sections were then processed as in Ward
300 et al. (2016), before examination under light microscopy for the presence of parasites and
301 pathologies using a Nikon Eclipse N*i*-E (Nikon). Digital images and measurements were
302 obtained using NIS-Elements imaging software (Nikon).

303 Post-fixation of glutaraldehyde-preserved digestive gland and mantle tissues was carried out
304 in 1% osmium tetroxide/0.1M sodium cacodylate buffer before processing as in Ward et al.
305 (2016). Grids were examined using a JEOL JEM1400 transmission electron microscope
306 (JEOL) and digital images captured using an AMT XR80 camera with AMT V602 software
307 (AMT Imaging).

308 *2.9* In situ *hybridisation (ISH)*

309 Digoxigenin (DIG)-labelled oligonucleotide probes targeting the 282 bp "SMART2" region of
310 the *Marteilia* 18S gene were synthesised from genomic DNA extracted from a *M. cocosarum*-
311 infected cockle using primers SS2 (Le Roux et al., 1999) and SAS1_MOD. Probes were
312 synthesised in 100 µl PCR reactions comprising 1× Green GoTaq Flexi Buffer, 2.5 mM $MgCl_2$,
313 0.2 µM each primer, 200 µM PCR DIG labelling mix (Roche, Switzerland), 2.5 U GoTaq Flexi
314 DNA polymerase, and 6 µl template DNA. Cycling conditions were as detailed in Le Roux et
315 al. (1999) for the SS2/SAS1 primer set. Resultant amplicons were visualised as above on 2%
316 agarose-TBE gels before probes were purified by the addition of 1× reaction volume of
317 AMPure XP sample purification beads, washed twice with 80% molecular-grade ethanol, and

318 eluted into 50 µl molecular-grade water. Probe was quantified using QuantiFlour ONE dsDNA
319 reagents on a Quantus fluorometer (both Promega) and normalised to a concentration of 10
320 ng/µl.

321 Whole animal sections of *Marteilia*-infected cockles collected in 2021, 2022 and 2009 (3 µm
322 thickness) were collected onto poly-L-lysine coated glass slides. Sections were dewaxed and
323 rehydrated with Xylene substitute (Thermo Fisher Scientific) twice for five minutes each,
324 followed by two washes in 100% ethanol for 4 minutes each. Sections were then washed once
325 in Tris-buffered saline (TBS) at pH 7.5 for 2 minutes. Next, sections were overlaid with 1 ml
326 Proteinase K in TBS pH 7.5 (45 µg/ml) for 20 minutes at 37°C, before washing in deionised
327 water for 5 minutes. Sections were then treated with ice-cold 12% acetic acid for 45 seconds
328 to increase permeability of the tissue, before dehydration by washing in 70% ethanol for 3
329 minutes, followed by 100% ethanol for a further 3 minutes. Sections were rinsed in 2× saline
330 sodium citrate (SSC) for 1 minute with agitation, before storage in TBS (pH 7.5). GeneFrames
331 (Thermo Fisher Scientific) were applied to each slide, and sections overlaid with 120 µl
332 hybridisation buffer (50% formamide, 4× SSC, 1× Denhardt's solution, 10% dextran sulphate,
333 250 µg/ml single-stranded DNA from salmon testes) containing 50 ng probe. For negative
334 control samples, no probe was added to the hybridisation buffer. Sections were denatured by
335 heating to 94°C for 6 minutes, before immediately cooling on ice for a further 6 minutes. Slides
336 were then hybridised overnight at 42°C in a humid chamber. The following morning,
337 GeneFrames were removed and sections washed with 3 times for 10 minutes each with wash
338 buffer (0.5× SSC, 6 M urea, 20 µg/ml BSA) pre-warmed to 42°C, before two further washes
339 for 5 minutes each in TBS pH 7.5. Sections were then overlaid with a milk blocking buffer (1×
340 TBS pH 7.5, 6% (*w/v*) non-fat milk powder (Sigma Aldrich)) for 1 hour at room temperature.
341 Following this, sections were washed twice for 5 minutes each in TBS pH 7.5, before
342 incubation overlaid with Anti-DIG-AP Fab fragments (Roche) diluted 1:300 in TBS pH 7.5 for
343 1 hour in darkness. To remove excess antibody, slides were washed 5 times for 10 minutes
344 each in TBS (pH 7.5), before transfer to TBS pH 9.5 for two minutes. Slides were then overlaid
345 with 5-bromo-4-chloro-3-indoyl-1-phosphate (BCIP) nitroblue tetrazolium (NBT) chromogenic
346 substrate (Roche), diluted 20 µl/ml in TBS pH 9.5, for up to 1 hour in darkness at room
347 temperature. After this time, sections were washed three times in TBS pH 9.5 for 3 minutes
348 each, followed by counterstaining with Bismarck Brown Y solution (5% in 35% ethanol) for 15
349 minutes. Sections were then gently rinsed in running tap water to remove excess counterstain,
350 and dehydrated by washes in 70% ethanol and 100% ethanol (30 seconds each), followed by
351 two rinses in xylene substitute for 1 minute each. Sections were then air-dried for a minimum
352 of 30 minutes, before cover slipping. Sections were then examined under light microscopy

using a Nikon Eclipse NI (Nikon, Japan) and images of labelled parasite cells in host tissue captured using NIS-Elements Imaging Software (Nikon, Japan).

3. Results

3.1 PCR prevalence of parasites in Wash cockles

All Wash cockle samples (n=385) were initially screened for paramyxids using a nested, 18S-targeted primer set, producing 198 amplicons (51%). The result of these PCR screens, including the number of PCR positive animals per site and per health status (i.e. moribund or healthy) is summarised in Table 3, and detailed below. Phylogenetic analysis of the resulting sequences found that 22 of these were 100% identical to 18S sequences for *M. cocosarum* from the Burry Inlet, Wales. We denote this sequence type "*Marteilia cocosarum* BI1" (Burry Inlet 1). 167 samples amplified a closely related but distinct *Marteilia* sequence type with no identical matches in the NCBI GenBank database, most closely related to *M. cocosarum* (sequence identity 99.4%). We denote this sequence type "*Marteilia cocosarum* WE1" (Wash Estuary 1). Nine samples produced mixed chromatogram traces which required cloning, and subsequent sequencing of cloned amplicons revealed both *M. cocosarum* BI1 and *M. cocosarum* WE1 to be amplified from these animals.

The highest proportion of *Marteilia*-positive animals were collected from the Mare Tail and Dills Sand sites, in samples collected in 2021 and 2022. A greater number of moribund animals were PCR positive than healthy animals at both Mare Tail (83.7% vs 34% in 2021) and Dills Sand (95% vs 42% in 2021). No healthy cockles were collected in 2022, but 100% of moribund animals from Dills Sand and 88% of moribund animals from Mare Tail were PCR positive. At Inner West Mark Knock, sampled in July 2021, 22% of moribund animals were PCR-positive, compared to 10% of healthy animals. Across all sites, there were significantly greater *Marteilia* positives (Chi-Sq p = 0.00001158) detected by PCR in moribund cockles compared to healthy cockles.

Table 3. Total *Marteilia cocosarum* prevalence in healthy vs. moribund cockles per site, detected via PCR screen.

Site	Sampling date	***Marteilia cocosarum* WE1**		***Marteilia cocosarum* BI1**		***Marteilia cocosarum* WE1 + BI1**		**Total *Marteilia cocosarum* positives**	
		Healthy	**Moribund**	**Healthy**	**Moribund**	**Healthy**	**Moribund**	**Healthy**	**Moribund**
Mare Tail	28/04/2021	17/50 (34%)	41/49 (84%)	0	0	0	0	**17/50 (34%)**	**41/49 (83.7%)**
	16/05/2022	NA	48/48 (100%)	NA	0	NA	0	**NA**	**48/48 (100%)**
Dills Sand	28/04/2021	14/50 (28%)	27/40 (67.5%)	6/50 (12%)	4/40 (10%)	1/50 (2%)	7/40 (17.5%)	**21/50 (42%)**	**38/40 (95%)**
	16/05/2022	NA	30/48 (62.5%)	NA	12/48 (25%)	NA	0	**NA**	**42/48 (88%)**
Inner West Mark Knock	26/07/2021	5/50 (10%)	10/50 (20%)	0	0	0	1/50 (2%)	**5/50 (10%)**	**11/50 (22%)**

PCR screens of cockle extracts samples collected in 2021 using a nested primer set targeting the 18S gene of haplosporidians produced a total of 158 amplicons (54%), with no significant difference in the total number of positives between healthy or moribund animals at any site. A greater number of haplosporidian positives were found from the sites sampled in April (Mare Tail (total 55/98); Dills Sand (total 66/90)) than Inner West Mark Knock, sampled in July (17/100). Sequencing of these amplicons produced a total of 128 usable sequences, with the remining 18 amplicons producing unusable, mixed chromatogram traces, likely indicating the amplification of more than one haplosporidian sequence type. In total seven haplosporidian sequence types were amplified, as summarised in Table 4. The most abundant haplosporidian lineage amplified across all three sites were *Minchinia* spp., including *M. tapetis*, a parasite of the grooved carpet shell *Ruditapes decussatus* also known to infect *Cerastoderma edule* in Spain (Carballal et al., 2020), *M. tapetis* was present at all sites, and in both healthy and moribund animals. Prevalence was highest at Dills Sand, with a similar percentage of positives between moribund (27.5%) and healthy (22%) animals. An uncharacterised *Minchinia* species, previously shown to have a strong association with *C. edule* populations in Spain (Ramilo et al., 2018), was also present at all sites, generally at a higher PCR prevalence in moribund animals. *Minchinia* sp. was amplified from 57%, 55% and 12% of moribund animals from Mare Tail, Dills Sand and Inner West Mark Knock, respectively. The parasite was also present in healthy animals from each site, at 26%, 42% and 4% prevalence, respectively.

Five other haplosporidian sequence types were also amplified from cockles, though at much lower prevalence. The *C. edule* parasite *Haplosporidium edule* was amplified from a low number of healthy cockles from Mare Tail and Dills Sand (4% and 2%, respectively), and a single moribund cockle (2%) from Inner West Mark Knock. The chiton parasite *Minchinia*

410 *chitonis* amplified from two healthy cockles collected at Mare Tail (4% prevalence), but no
411 other site. A previously detected but uncharacterised sequence type, originally amplified from
412 water and sediment collected in the Fleet Lagoon, Dorset, UK, was amplified from a single
413 healthy cockle from Mare Tail. A second environmental sequence type, originally detected in
414 water samples collected close to the Mumbles Pier, Swansea, UK, was also amplified from a
415 single healthy cockle at Mare Tail. A novel sequence type was amplified from a single cockle,
416 also a healthy individual collected at Mare Tail. This sequence has no close characterised
417 relatives, though showed high identity (99%) to an uncharacterised lineage previously
418 amplified from incubation water associated with the crab species *Carcinus maenas* and
419 *Cancer pagurus* collected in Newton's Cove, Dorset, UK.

420 **Table 4.** Total Haplosporidian prevalence in healthy vs. moribund cockles per site, detected via PCR screen.

Site	Sampling Date	Health status (total animals)	*Minchinia tapetis*	*Minchinia* sp. ex. *Cerastoderma edule*	*Minchinia chitonis*	*Haplosporidium edule*	Coastal water clone KF208577 (Fleet Lagoon)	Coastal water clone MT334478 (Mumbles Pier)	Novel sequence type 01
Mare Tail	28/04/2021	Moribund (49)	2 (4%)	28 (57%)	0	0	0	0	0
		Healthy (50)	5 (10%)	13 (26%)	2 (4%)	2 (4%)	1 (2%)	1 (2%)	1 (2%)
Dill's Sand	28/04/2021	Moribund (40)	11 (27.5%)	22 (55%)	0	0	0	0	0
		Healthy (50)	11 (22%)	21 (42%)	0	1 (2%)	0	0	0
Inner West Mark Knock	26/07/2021	Moribund (50)	5 (10%)	6 (12%)	0	1 (2%)	0	0	0
		Healthy (50)	3 (6%)	2 (4%)	0	0	0	0	0

421

422 *3.2 Histopathology,* in situ *hybridisation and Transmission Electron Microscopy (TEM) of*
423 *Marteilia cocosarum*

424 *Marteilia*-like cells were observed within the connective tissues, most commonly observed in
425 the gill and mantle tissues of 40% of the cockles sampled from Mare Tail and 37% of cockles
426 sampled from Dills Sand. *Marteilia*-like cells presented with the typical cell within cell
427 arrangement associated with paramyxid infections and were often associated with large focal
428 areas of haemocytic infiltration (Fig 1). In the case of systemic infections *Marteilia*-like cells
429 were observed within the haemal sinuses and the vesicular connective tissues surrounding
430 the digestive gland and gonad but were not observed within the epithelial cells of the digestive
431 gland tubules and stomach tissues. ISH labelling confirmed presence of *Marteilia*-like cells
432 within areas of pronounced haemocytic infiltration, especially in the connective tissues of the
433 gills and mantle, parasite cells staining a deep blue colour (Fig 2).

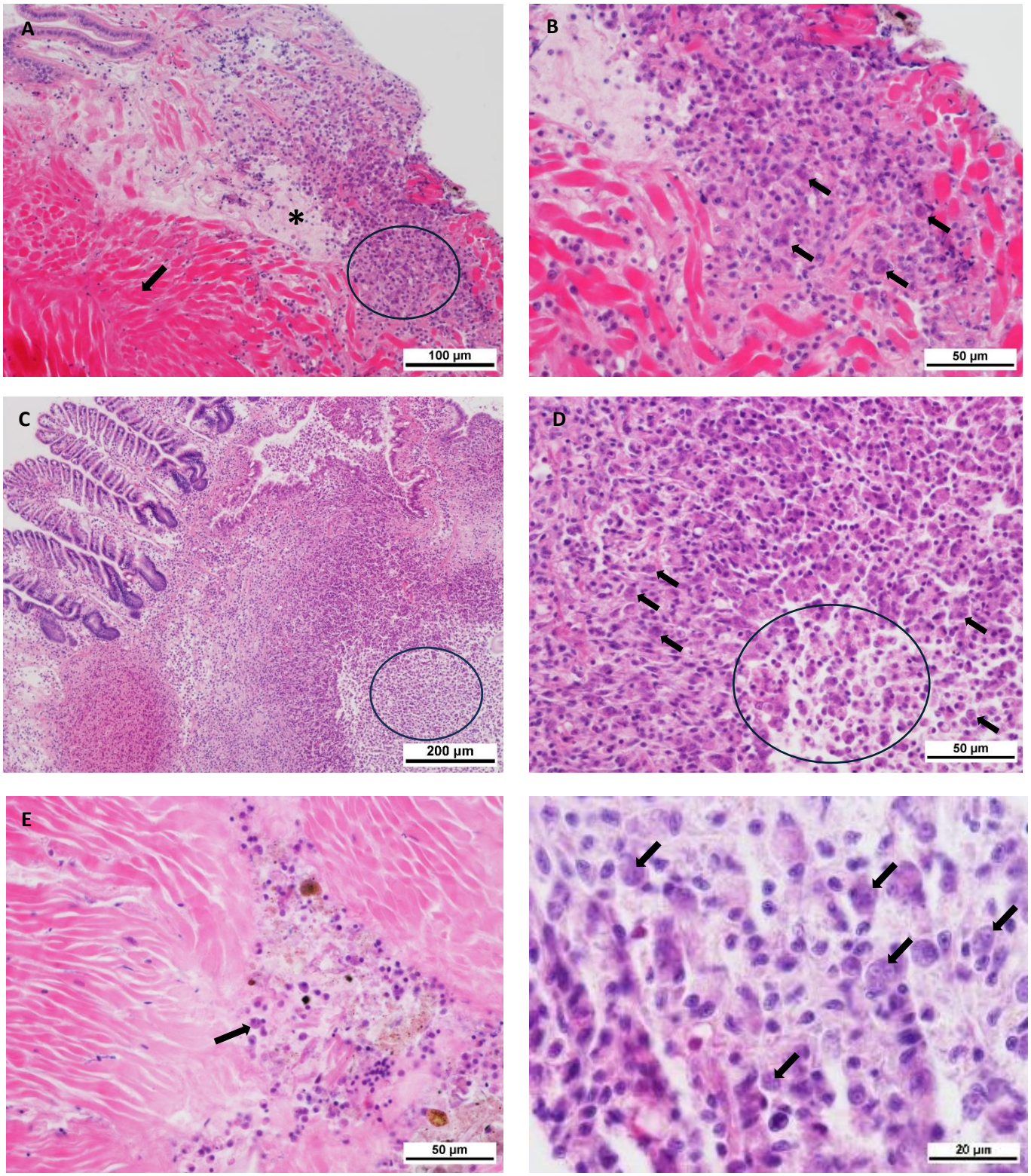

Figure 1. A) Muscle (arrow) and vesicular connective tissues (*) of *C. edule* displaying a focal area of haemocytic infiltration (circle). Scale bar = 100 µm. B) Higher magnification of 'A', focal area of haemocytic infiltration containing large multinucleate cells (arrows), cell within cell arrangement can be observed which is typical of paramyxid infections such as *Marteilia* sp. Scale bar = 50 µm. C) Marked host response observed in the Gill area with dense haemocyte infiltration containing multinucleate *Marteilia*-like cells, tissue necrosis (circled area) was also observed. Scale bar = 200 µm. D) Higher magnification of 'C' cellular inflammation, large multicellular *Marteilia*-like cells (arrows) are visible throughout the lesion. Area of necrosis (circle) shows sloughing, rounding and dissociating cells associated with apoptosis and necrosis. Scale bar = 50 µm. E) *Marteilia*-like cells (arrow) observed in the sinus of the adductor muscle. Scale bar = 50 µm. F) Cell within cell appearance of *Marteilia*-like cells (arrows) in connective tissues of gill. Scale bar = 20 µm. All images H&E stained.

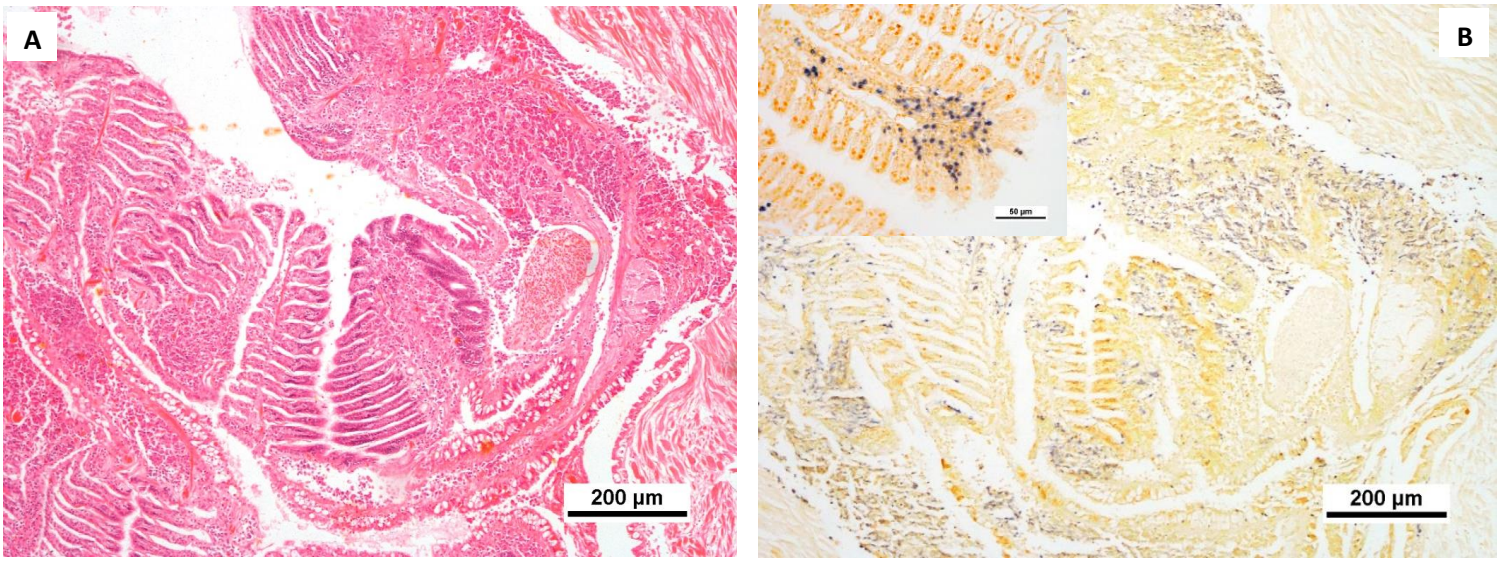

Figure 2. *In situ* hybridisation (ISH) of *Marteilia*- like parasites within *C. edule* tissues. A) Negative control gill tissues. H&E stain. B) *In situ* hybridisation (ISH) of the same region of gill tissues, labelled with Digoxigenin (DIG)-labelled Smart-2-probe targeting the 282 bp region of the Marteilia 18S gene. Blue stain highlights presence of *Marteilia*- like parasites within the connective tissues and associated with pronounced areas of haemocytic infiltration. Scale bars = 200 µm. Inset displaying magnified region of gill tissue infected by *Marteilia*-like pathogens. Scale bar = 50µm.

As described by Skujina *et al*. (2022) primary cells appeared to contain up to three tertiary cells, no spore like formations were noted. TEM analysis found multiple *Marteilia*-like cells distributed throughout the connective tissues, clearly displaying the characteristic cell within cell arrangement of the secondary and tertiary cells within the primary cell (Fig. 3). Haplosporosome-like structures were observed within primary and secondary cells only. Distinct double membranes were observed separating the primary, secondary and tertiary cells, primary cell membrane denser in appearance when compared to secondary and tertiary cells. Interestingly the cell membrane of the primary cell appeared to possess pore like structures, small discrete areas where the membrane appeared as a single membrane, it is not clear whether these areas may be involved in endocytosis between the host cells and parasite. Higher levels of infection were observed in moribund animals when compared to healthy animals at both sites, 60% and 63% prevalence in moribund animals and 24% and 12% in healthy animals at Mare Tail and Dills Sand respectively.

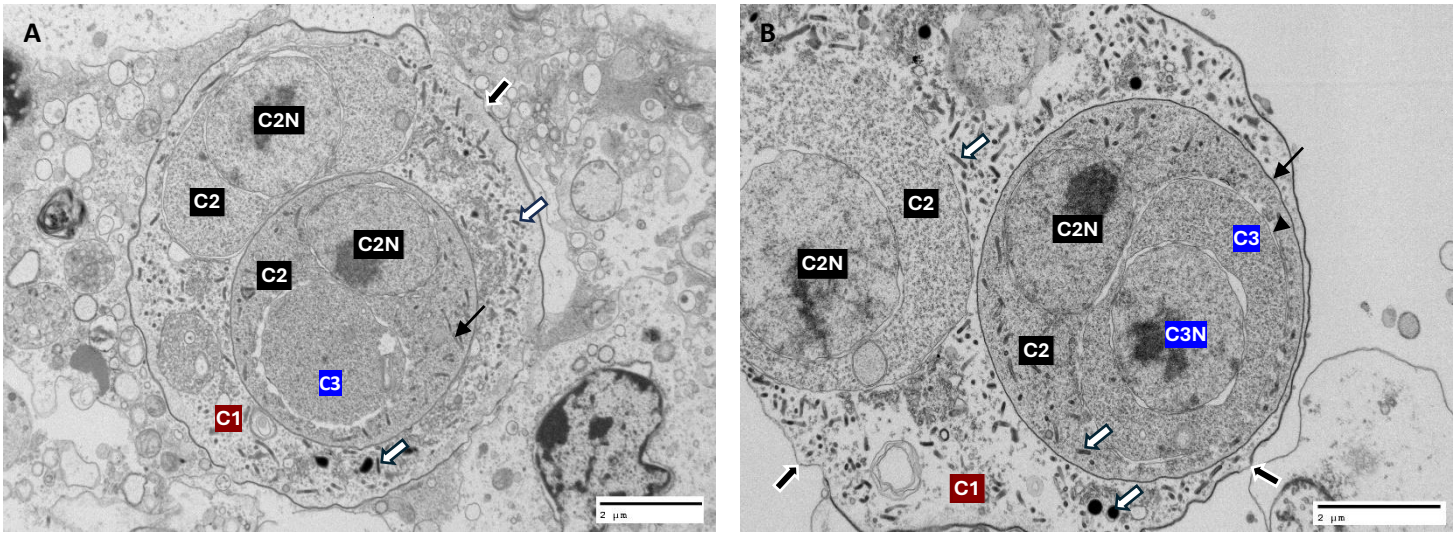

Figure 3. Transmission electron microscopy of *Marteilia*-like cells within *C. edule* tissues. A) Single primary cell (C1) containing multiple rod shaped haplosporosomes (white arrow) and two secondary cells (C2) containing clear nuclei (C2N). Some secondary cells contained haplosporosomes (line arrow) and tertiary cells (C3). Note the pores within the primary cell membrane (black arrow). B) Higher magnification detailing secondary and tertiary cells. Primary cell (C1) contains two secondary cells (C2) both of which display clear nuclei (C2N). Secondary cells are bound within a clear membrane (line arrow) and can contain haplosporosomes (white arrows). Tertiary cell (C3) also contains a clear nucleus (C3N) and is bound within a clear membrane (arrowhead). Haplosporosomes were not observed within the tertiary cells. Note the appearance of single membrane in certain areas of the primary cell membrane (black arrows). Scale bars = 2 µm.

Cockles at both sites showed multiple co-infections with various different pathogens by histology, summarised see Table 5 for summary of histology results. *Nematopsis* sp. is a common infection of cockles and was observed at both sites during this study, although higher level prevalence was reported at Mare Tail. Rickettsia-like organisms were observed within the digestive gland at both sites at similar prevalence and a bacterial infection was noted in some cockles. This appeared to be seen more frequently in moribund animals and was associated with necrosis of the tissues so is likely a secondary infection. Necrosis and inflammation was observed in a large number of animals samples from Mare Tail when compared to Dills Sand. Inflammation was commonly associated with presence of Marteilia-like cells. Haemocytic neoplasia was observed in a few cockles at each site, the majority thought to be type B (Carballal et al., 2001; Iglesias., 2006; Metzger et al., 2006). *Bucephalus minimus* was observed at low level at both sites in healthy and a few moribund animals at Dills Sand. *Trichodina* sp. were also observed at both sites in healthy animals and a few moribund animals at Mare Tail. Low levels of Turbellaria sp., digenean and ciliate species were observed at both sites.

496 **Table 5.** Summary of pathogens and pathology observed in healthy and moribund cockles, and total cockles examined
497 histologically at each site.

Site and condition	Marteilia -like cells	Nematopsis sp.	RLO/CLO	Trichodina sp.	Bucephalus sp.	Turbellaria sp.	Digenean	Ciliates	Bacteria	Haemocytic neoplasia	Inflammation	Necrosis
Mare Tail												
Moribund cockles (49)	60%	88%	15%	22%	0	10%	0	1%	25%	3%	78%	28%
Healthy cockles (50)	24%	60%	14%	14%	16%	2%	2%	0	0	0	24%	0
Total cockles (99)	40%	72%	14%	18%	9%	6%	1%	0	11%	1%	37%	12%
Dills Sand												
Moribund cockles (40)	63%	24%	6%	0	2%	0	0	0	4%	0	0	0
Healthy cockles (50)	12%	66%	16%	12%	8%	6%	4%	4%	0	6%	42%	0
Total cockles (90)	37%	45%	11%	6%	5%	3%	0	2%	2%	3%	21%	0

498

499

500

501 *3.3 Molecular analyses and phylogeny of* Marteilia *in the Wash*

502 The Wash *Marteilia* lineage (*M. cocosarum* WE1) can be phylogenetically distinguished from
503 the closely-related genotype *M. cocosarum* BI1 and more distantly related sister species *M.*
504 *cochillia* using a relatively short (approx. 500 bp) region of the 18S gene of the rRNA array,
505 using Sanger sequencing. In this region, *M. cocosarum* WE1 shows five consistent nucleotide
506 differences to *M. cocosarum* BI1, and four in comparison to *M. cochillia* (Table 6). To further
507 discriminate *M. cocosarum* WE1, additional PCRs and high-throughput sequencing were
508 undertaken to retrieve sequence data for the full transcribed rRNA array. Across the full array,
509 the two *M. cocosarum* lineages show 99.83% similarity, with 14 consistent nucleotide
510 differences (Table 6). Though the ITS regions (ITS1 and ITS2) typically show higher rates of
511 variation between closely related lineages, comparison of these regions showed there to be
512 only one nucleotide difference between *M. cocosarum* WE1 and *M. cocosarum* BI1 in ITS1
513 (681 bp), and five differences within the ITS2 (781 bp), the same number of consistent
514 differences observed within the 18S gene.

515

516

517 **Table 6:** Nucleotide differences and percentage similarity across the different gene regions of the ribosomal rRNA
518 array between *Marteilia* lineages. Sizes of each region of the array are given.

Lineages compared	Nucleotide differences/percentage similarity						% similarity over full transcribed array
	ETS 1,547 bp	18S 1,792 bp	ITS1 681 bp	ITS2 781 bp	28S 3,471 bp	Full transcribed array 8,414 bp	
M. cochillia ~ *M. cocosarum* WE1	13 (99.16%)	3 (99.83%)	6 (99.12%)	6 (99.23%)	9 (99.74%)	37	99.56
M. cochillia ~ *M. cocosarum* BI1	15 (99.03%)	4 (99.78%)	6 (99.12%)	9 (99.85%)	10 (99.71%)	44	99.48
M. cocosarum BI1 ~ *M. cocosarum* WE1	2 (99.87%)	5 (99.72%)	0 (100%)	5 (99.36%)	1 (99.97%)	13	99.85

519

520 Comparatively, *M. cocosarum* WE1 was 99.56% similar to *M. cochillia* from Spanish cockles,
521 with 37 nucleotide differences across the full array. The greatest sequence divergence
522 between these two lineages is within the ITS1 with six nucleotide differences (99.12%
523 sequence identity) and ETS with 13 nucleotide differences (99.16% sequence identity).
524 Between the two *M. cocosarum* lineages, only two nucleotide differences exist within the ETS
525 region, and one within the ITS1 region. Given this level of sequence similarity, the 18S gene
526 or ITS2 regions appear to be most suitable for discriminating between the three lineages
527 (Table 6), although an inconsistent indel in the ITS2 of *M. cocosarum* (Skujina et al. 2022)
528 makes this region difficult to use as a diagnostic marker, due to the requirement to perform
529 confirmatory sequencing. Therefore, we suggest the 18S gene as the most appropriate option
530 for discrimination between lineages. Full transcribed rRNA arrays were deposited to GenBank
531 under accession numbers PP853058- PP853071.

532 Due to 100% sequence identity across all 18S sequences generated in this study for *M.*
533 *cocosarum* WE1, a single representative sequence was used for phylogenetic analysis.
534 Bayesian phylogenetic analysis shows that *M. cocosarum* WE1 and BI1 branch as sister
535 clades with very high posterior probability support (0.99) (Fig. 4). The *M. cocosarum* WE1 +
536 BI1 clade branches as sister to *M. cochillia*, though this relationship receives lower statistical
537 support (posterior probability 0.90). In phylogenetic analyses of the full ribosomal arrays of *M.*
538 *cocosarum* WE1 (n=13), BI1 (n=6) and *M. cochillia* (n=6), using *M. octospora* as an outgroup,
539 each lineage forms a mutually exclusive, monophyletic clade with maximal statistical support
540 (posterior probability = 1.0) (Fig. 5). *M. cocosarum* WE1 and *M. cocosarum* BI1 group as sister
541 clades (with maximal support), clearly distinguishing the two lineages as independent
542 genotypes, with *M. cocosarum* lineages in turn grouping distinctly from *M. cochillia* from
543 Spanish cockles.

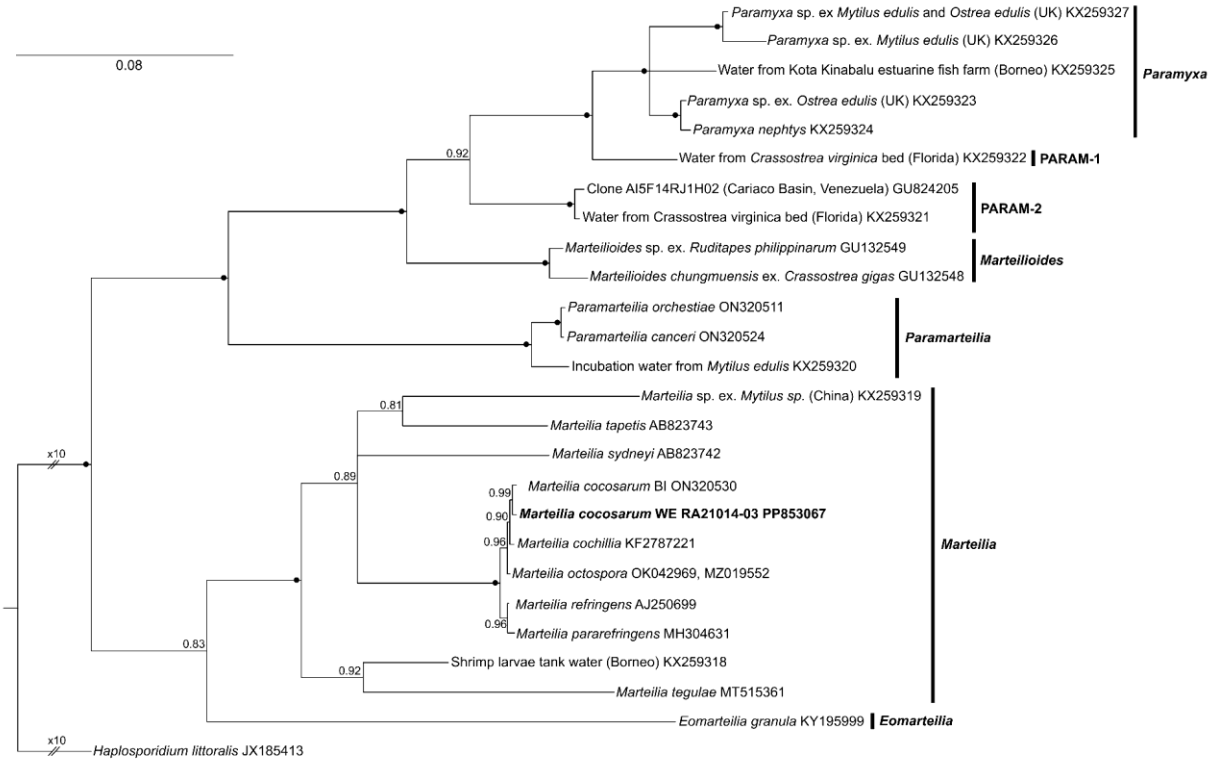

Figure 4. A phylogenetic comparison of closely related Paramyxid lineages including *M. cocosarum* WE1 (in bold) and *M. cocosarum* BI1 both infecting *C. edule* in the UK, as well as *M. cochillia* known to infect *C. edule* in Spain. Phylogenetic tree constructed using Bayesian analysis using 18S region sequences of the rRNA array and *Haplosporidium littoralis* as an outgroup. Maximal Bayesian Posterior Probabilities support values are labelled above branches. Accession numbers are detailed following the species/sample name or ID.

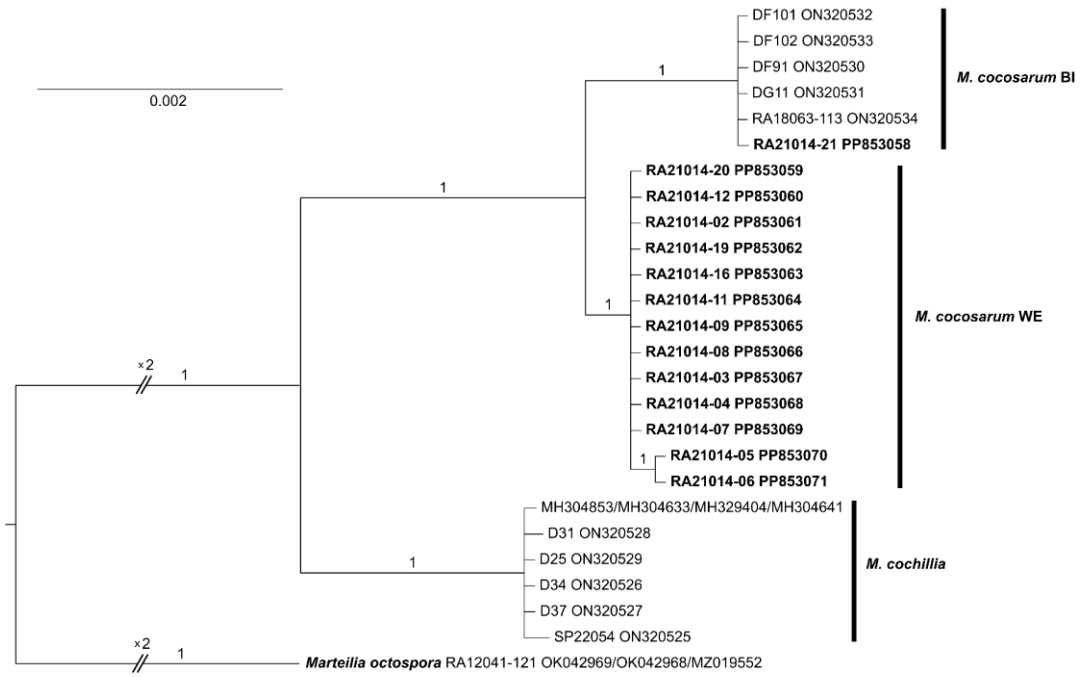

Figure. 5. A phylogenetic comparison of *Marteilia cocosarum* WE infecting *C. edule* in the Wash estuary, to *Marteilia cocosarum* BI infecting *C. edule* in the Bury Inlet, and their relationship to *Marteilia cochillia* infecting *C. edule* in Spain. Based upon Bayesian analysis of rRNA sequences with *Marteilia octospora* as an outgroup.

556 **4. Discussion**

557 The present study provides evidence of a novel genotype of the paramyxid parasite *Marteilia*
558 *cocosarum* infecting common cockles *Cerastoderma edule* in The Wash Estuary, UK, which
559 we denote *M. cocosarum* WE1 (Wash Estuary 1). Prevalence of the parasite, using both
560 molecular and histopathological screening techniques, was observed to be significantly higher
561 in moribund, surface-bound cockles than healthy, buried animals. Reanalysis of archive
562 samples collected at sites within The Wash experiencing mortalities in 2009 using microscopy
563 and molecular techniques shows the parasite to have been present within cockle populations
564 within the estuary since at least May 2009, shortly after mortalities were first documented in
565 2008.

566 Molecular and histopathological analyses confirm this lineage to show high identity to *M.*
567 *cocosarum* BI1 (Burry Inlet 1), from the Burry Inlet, Wales, both genetically and in its pathology
568 and mode of infection. *M. cocosarum* genotypes are demonstrably distinct phylogenetically
569 and histopathologically from their closest known relative *M. cochillia*, which infects *C. edule* in
570 Spain. Analysis of full rRNA array sequences allows the two *M. cocosarum* genotypes to be
571 distinguished by consistent nucleotide differences in several regions, with the two lineages
572 showing high sequence identity to each other across the rRNA array (99.83%), and lower
573 similarity to *M. cochillia* (99.56%). The ETS within the intergenic spacer region showed the
574 greatest sequence divergence between *M. cocosarum* and *M. cochillia*, but low variation was
575 observed between the two *M. cocosarum* genotypes (15 nucleotide differences compared to
576 two over the 1,547 bp region). To distinguish between *M. cocosarum* WE1 and *M. cocosarum*
577 BI1, we recommend sequences of the 18S gene, which shows five consistent nucleotide
578 differences between the two *M. cocosarum* genotypes, and three to four nucleotide differences
579 between *M. cochillia and M. cocosarum,* dependent on genotype (Table 6). Although ITS2
580 shows a larger number of consistently variable nucleotides, an inconsistent indel in this region
581 makes it difficult to retrieve consistent quality sequences without the need for amplicon cloning
582 (Skujina et al 2022).

583 Histopathological examination showed that *M. cocosarum* WE1 infections are consistent with
584 those observed for *M. cocosarum* BI1. Parasite cells are observed throughout the gill and
585 mantle tissues, and within the connective tissues of the adductor muscle and digestive gland
586 (but not within the digestive gland tubules themselves) in heavily infected animals. Both
587 genotypes also elicit a host response, with inflammatory regions containing haemocytic
588 infiltration observed in infected individuals. This tissue tropism differs from that observed with
589 other *Marteilia* species, including *M. cochillia*, which are typically found within the epithelium
590 of the digestive gland tubules, between digestive epithelial cells of the digestive ducts, and
591 within the intestinal lumen (Carrasco et al. 2015; Villalba et al., 2014).

The initial characterisation of *M. cocosarum* followed widespread mortalities of *C. edule* in the Burry Inlet, Wales, and within populations along the Welsh coast. Although the parasite was detected at high PCR prevalence, and high levels of infection in individual animals was observed by histopathology, across the sites surveyed, only a tentative link between parasite presence and mortality could be made, with the parasite implicated alongside a number of additional stressors (Skujina et al. 2022). These conclusions were made primarily due to a lack of mortality data for many sites, and incomplete sampling limiting histopathological and electron microscope examination of affected cockles. Furthermore, cockles collected during the Welsh study were not recorded as either moribund or "healthy" at the time of sampling, and therefore the correlation between *M. cocosarum* presence and cockle health status could not be explored. In our present sample set, a strong association between cockle moribundity and *Marteilia* infection can be made: weak, gaping cockles on the sediment surface show significantly higher infection prevalence and intensity than those buried within the sediment and showing no clinical disease signs.

Marteilia prevalence in moribund cockles was lower at Inner West Mark Knock (IWMK) compared to the other two sites surveyed (22% vs 95% and 83.7% for Dills Sand and Mare Tail in 2021, respectively). Prevalence was always higher in moribund relative to healthy cockles. The extent of mortalities on IWMK in 2021 did not appear to be dissimilar to that of Dills Sand or Mare Tail, with regards to losses in cockle numbers and biomass. Equally, IWMK was sampled later on in the summer season than the other two sites, when temperatures were likely to be warmer and therefore *Marteilia* prevalence would be greater due to increased stress. Yet, the lack of *Marteilia* prevalence here strongly suggests that additional agents are involved in the cockle mortalities observed. It is important to consider the influence of other stressors on cockle populations, particularly across hydrographically distinct areas of The Wash estuary. Exposure to varying biotic (e.g. cohabiting benthic invertebrates, other parasites) and abiotic (e.g. chemical exposure or nutrient limitation) conditions may reduce overall host fitness, resulting in increased susceptibility to parasite infection. The involvement of planktonic crustaceans as intermediate hosts has long been considered to play a role in the life cycle of *Marteilia* species (Audemard et al., 2002; Arzul et al., 2014), and so consideration may be given to the composition of benthic and planktonic invertebrate communities across The Wash.

Histopathology identified disseminated neoplasia in all three populations surveyed. Prevalence was highest at Inner West Mark Knock, with the condition observed in 16 individuals, 15 of which were moribund animals, and one was a healthy animal. Disseminated neoplasia is a leukaemia-like cancer reported in at least 15 species of marine bivalves, including clams, mussels, oysters and cockles (Metzger et al., 2016; Diaz et al., 2011).

Typically, the disease is characterised by the presence of large, anaplastic cells within the haemolymph. Two different types of neoplastic cells were observed within the Wash cockles, described as Type A and B, characterised by the shape of nuclei and arrangement of cells and distinguished phylogenetically as two distinct cancer lineages (Carballal et al., 2001; Metzger et al., 2016). The majority presented signs of Type B neoplasia (12 individuals), whilst 4 individuals were observed to have Type A. In diseased animals, the cells multiply and reduce the number of normal haemocytes, eventually resulting in the death of the host (Barber., 2004). It is therefore possible that the higher incidence of disseminated neoplasia observed at Inner West Mark Knock, where *Marteilia* prevalence was comparatively low compared to the other two sites surveyed, may be linked to the atypical mortalities observed at this site. Disseminated neoplasia in cockles has also been identified in at least one site in Wales (Bruzos et al., 2023), but its link to the mortalities occurring in this region, to our knowledge, has not been investigated.

Cockles were also screened by PCR for the presence of haplosporidian parasites, after previous studies suggested a link between a number of haplosporidian species and cockle declines in the Burry Inlet (Elliott et al. 2012; Longshaw & Malham 2013). Two lineages – *Minchinia tapetis* and an uncharacterised *Minchinia* sp. closely related to *M. mercenariae* – were amplified, with both showing higher prevalence in moribund than apparently healthy cockles. However histopathological screening detected only a single haplosporidian infection at low intensity in a healthy animal from Mare Tail (Table 5). This same animal was PCR-positive for *M. tapetis*. Both *Minchinia* lineages amplified in this study have been previously reported to be present in cockle populations in Europe. *M. tapetis* has been observed infecting *C. edule* in Galicia, Spain, where infection prevalence appeared high, but intensity within infected individuals was low (Carballal et al. 2020). Seasonal studies in Spain showed infection prevalence to be linked to seawater temperature, with infection highest in the warmer months. In our study, molecular prevalence of the parasite was highest in animals sampled from Dills Sand in April, however as no repeat sampling was conducted at the same sites in the summer it cannot be determined whether the prevalence of observable (histopathological) infection is higher among cockles in The Wash estuary during the warmer months. The second *Minchinia* lineage amplified in this study is also known to occur within *C. edule* populations within Galicia. *Minchinia* sp. appears in cockles at a lower prevalence, and infections were of moderate or high intensity, with haplosporidian cells observed throughout the connective tissues in the digestive gland, gills and gonad. A strong host response is observed, though to date is not associated abnormal cockle mortalities. Uni- and bi-nucleate parasite stages and multinucleate plasmodia were observed in infected individuals, but no spore stages could be seen, preventing conclusive identification of the parasite (Ramilo et al. 2018). However, given

the high sequence similarity of this lineage to *M. mercenariae*, it is likely that this is the same parasite previously reported associated with cockle populations experiencing abnormal mortalities and population declines, including those in the Burry Inlet (Elliott et al. 2012; Longshaw & Malham, 2013). As no link between haplosporidian infection and cockle moribundity could be made at any site surveyed in this study, and that none of the haplosporidian lineages found in The Wash have been demonstrably linked to increased or unusual cockle mortalities, it is unlikely that these parasites are a primary cause of mortalities within Wash cockles.

5. Conclusion

The combined analyses from the present study describe a novel genotype of *M. cocosarum* WE1 infecting cockles in The Wash estuary. This discovery follows the recent description of a closely related genotype of the same parasite *M. cocosarum* BI1 causing marteiliosis in UK cockles in the Burry Inlet, Wales (Skujina et al., 2022). We also provide evidence that *M. cocosarum* has been present in cockle populations within The Wash since at least May 2009, which is shortly after documented evidence of mortalities and population declines were reported in 2008. Further work is required to understand the geographic extent of the parasite within The Wash and surrounding areas, including cockle fisheries in the northeast and the Thames catchment. Also important is understanding the risk of transmission of the parasite to new locations, particularly through intentional or accidental translocation of bivalve species between production areas. Further work should also aim to understand any differences in pathogenicity between this novel genotype compared to *M. cocosarum* BI1, as well as the biotic and abiotic factors which may contribute to high parasite prevalence and increased likelihood of infection outbreak.

6. Acknowledgements

This study was funded by Defra project AHPFFB2: Diagnostics and Emerging Disease**.**

7. References

Arzul I, Chollet B, Boyer S, Bonnet D, Gaillard J, Baldi Y, Robert M, Joly JP, Garcia C, Bouchoucha M (2014) Contribution to the understanding of the cycle of the protozoan parasite *Marteilia refringens*. Parasitology 141(2): 227-240

Audemard C, Le Roux F, Barnaud A, Collins C, Sautour B, Sauria PG, de Montaudouin X, Coustau C, Combes C, Berthe F (2002) Needle in a haystack : involvement of the copepod *Paracartia grani* in the life-cycle of the oyster pathogen *Marteilia refringens*. Parasitology 124(3): 315-323.

Barber, B.J., (2004). Neoplastic diseases of commercially important marine bivalves. *Aquatic Living Resources*, *17*(4), pp.449-466.

Bruzos AL, Santamarina M, García-Souto D, Díaz S, Rocha S, Zamora J, Lee Y, Viña-Feás A, Quail MA, Otero I, Pequeño-Valtierra A, Temes J, Rodrigues-Castro J, Aramburu L, Vidal-Capón A, Villanueva A, Costas D, Rodrígues R, Prieto T, Tomás L, Alvariño P, Alonso J, Cao A, Iglesias D, Carballal MJ, Amaral AM, Balseiro P, Calado R, El Khalfi B, Izagirre U, de Montaudouin X, Pade NG, Probert I, Ricardo F, Ruiz P, Skazina M, Smolarz K, Pasantes JJ, Villalba A, Ning Z, Ju YS, Posada D, Demeulemeester J, Baez-Ortega A, Tubio JMB. (2023). Somatic evolution of marine transmissible leukaemias in the common cockle *Cerastoderma edule.* Nature Cancer 4: 1575-1591.

Burdon D, Callaway R, Elliott M, Smith T, Wither A (2014) Mass mortalities in bivalve populations: a review of the edible cockle *Cerastoderma edule* (L). Estuar. Coast. Shelf Sci. 150: 271 – 280.

Carballal, M.J., Iglesias, D., Santamarina, J., Ferro-Soto, B. and Villalba, A., (2001). Parasites and pathologic conditions of the cockle Cerastoderma edule populations of the coast of Galicia (NW Spain). *Journal of Invertebrate Pathology*, *78*(2), pp.87-97.

Carballal MJ, Cao A, Iglesias D, González AI, Villalba A. (2020). Temporal dynamics of infection of cockles *Cerastoderma edule* with the protistan parasite *Minchinia tapetis* (Rhizaria: Haplosporida) in Galicia (NW Spain). J. Invertebr. Pathol. 172: 107349

Carrasco N, Andree KB, Lacuesta B, Roque A, Rodgers C, Furones MD. (2012). Molecular characterization of the *Marteilia* parasite infecting the common edible cockle *Cerastoderma edule* in the Spanish Mediterranean coast: a new *Marteilia* species affecting bivalves in Europe? Aquaculture 324: 20 – 26.

Carrasco N, Hine PM, Durfort M, Andree KB, Malchus N, Lacuesta B, González M, Roque A, Rodgers C, Furones MD. (2013). *Marteilia cochillia* sp. nov., a new *Marteilia* species affecting the edible cockle *Cerastoderma edule* in European waters. Aquaculture 412-413: 223 – 230.

Carrasco N, Green T, Itoh N. (2015). *Marteilia* spp. parasites in bivalves: a revision of recent studies. J. Invertebr. Pathol. 131: 143-157.

De Montaudouin X, Arzul I, Cao A, Carballal MJ, Chollet B, Correira S, Cuesta J, Culloty S, Daffe G, Darriba S, Diaz S, Engelsma M, Freitas R, Garcia C, Goedknegt A, Gonzales P, Grade A, Groves E, Iglesias D, Jensen KT, Joaquim S, Lynch S, Magalhaes L, Mahony K, Maia F, Malham S, Matias D, Nowaczyk A, Ruano F, Thieltges D, Villalba A. (2021). Catalogue of parasites and diseases of the common cockle *Cerastoderma edule*. UA Editora-Universidade de Aveiro. https://doi.org/10.34624/9a9c-9j21

Díaz, S., Renault, T., Villalba, A. and Carballal, M.J., (2011). Disseminated neoplasia in cockles Cerastoderma edule: ultrastructural characterisation and effects on haemolymph cell parameters. *Diseases of aquatic organisms*, *96*(2), pp.157-167.

Díaz S, Iglesias D, Villalba A, Carballal MJ. (2016). Long-term epidemiological study of disseminated neoplasia of cockles in Galivia (NW Spain): temporal patterns at individual and population levels, influence of environmental and cockle-based factors and lethality. J. Fish Dis. 39(9): 1027-1042. https://doi.org/10.1111/jfd.12436

Drummond, A.J. and Rambaut, A., (2007). BEAST: Bayesian evolutionary analysis by sampling trees. *BMC evolutionary biology*, *7*, pp.1-8.

Eastern IFCA. (1992). The Wash Fishery Order 1992 - Eastern IFCA (eastern-ifca.gov.uk)

Eastern IFCA. (2019). 2019 WFO cockle stock assessment. 2019 WFO (eastern-ifca.gov.uk).

Eastern IFCA. (2021). https://www.eastern-ifca.gov.uk/wp-content/uploads/2022/01/2021_09_29_Wash_Several_Fishery_management_plan_v4_agreed_at_45_EIFCA_Meeting.pdf [Accessed May 2024]

Eastern IFCA. (2022). Wash intertidal cockle survey report (2022). 2022_Wash_Intertidal_Cockle_Survey_Report.pdf (eastern-ifca.gov.uk)

Elliott, M., Burdon, D., Callaway, R., Franco, A., Hutchinson, T., Longshaw, M., Malham, S., Mazik, K., Otto, Z., Palmer, D. and Firmin, C., (2012). Burry Inlet cockle mortalities investigation 2009–2011. *University of Hull, Hull, UK*.

Garcia C, Mesnil A, Tourbiez D, Moussa M, Dubreuil C, Gonçalves de Sa A, Chollet B, Godfrin Y, Dégremont L, Serpin D, Travers M-A. (2021). *Vibrio aestuarianus* subsp. *cardii* subsp. nov., pathogenic to the edible cockles *Cerastoderma edule* in France, and establishment of *Vibrio aestuarianus* subsp. *aestuarianus* subsp. Nov. and *Vibrio aestuarianus* subsp. *francensis* subsp. nov. Int. J. Syst. Evol. Microbiol. 71(2):004654. https://doi.org/10.1099/ijsem.0.004654

Hartikainen, H., Ashford, O.S., Berney, C., Okamura, B., Feist, S.W., Baker-Austin, C., Stentiford, G.D. and Bass, D., (2014). Lineage-specific molecular probing reveals novel diversity and ecological partitioning of haplosporidians. *The ISME journal*, *8*(1), pp.177-186.

Hooper C, Ward GM, Foster R, Skujina I, Ironside JE, Berney C, Bass D. (2023). Long amplicons as a tool to identify variable regions of ribosomal RNA for improved taxonomic resolution and diagnostic assay design in microeukaryotes: using Ascetosporea as a case study. Front. Ecol. Evol. 11: 1266151.

Iglesias, D., (2006). Estudio patológico de las poblaciones de berberecho Cerastoderma edule (L.) de Galicia. *PhD diss., University of Vigo*.

Katoh K., Standley D. M. (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol. Biol. Evol.* 30, 772–780. doi: 10.1093/molbev/mst010

Kerr, R., Ward, G.M., Stentiford, G.D., Alfjorden, A., Mortensen, S., Bignell, J.P., Feist, S.W., Villalba, A., Carballal, M.J., Cao, A. and Arzul, I., 2018. Marteilia refringens and Marteilia pararefringens sp. nov. are distinct parasites of bivalves and have different European distributions. *Parasitology*, *145*(11), pp.1483-1492.Larsson, A. (2014): AliView: a fast and lightweight alignment viewer and editor for large datasets. Bioinformatics 30(22): 3276–3278.

Le Roux, F., Audemard, C., Barnaud, A. and Berthe, F., 1999. DNA probes as potential tools for the detection of Marteilia refringens. *Marine Biotechnology*, *1*, pp.588-597.

Longshaw, M. and Malham, S.K., (2013). A review of the infectious agents, parasites, pathogens and commensals of European cockles (Cerastoderma edule and C. glaucum). *Journal of the Marine Biological Association of the United Kingdom*, *93*(1), pp.227-247.

Messing, J., (1983). [2] New M13 vectors for cloning. In *Methods in enzymology* (Vol. 101, pp. 20-78). Academic Press.

Metzger, M.J., Villalba, A., Carballal, M.J., Iglesias, D., Sherry, J., Reinisch, C., Muttray, A.F., Baldwin, S.A. and Goff, S.P., (2016). Widespread transmission of independent cancer lineages within multiple bivalve species. *Nature*, *534*(7609), pp.705-709.

Metzger, M.J., Villalba, A., Carballal, M.J., Iglesias, D., Sherry, J., Reinisch, C., Muttray, A.F., Baldwin, S.A. and Goff, S.P., (2016). Widespread transmission of independent cancer lineages within multiple bivalve species. *Nature*, *534*(7609), pp.705-709.

Miller, M.A., Pfeiffer, W. and Schwartz, T., (2010), November. Creating the CIPRES Science Gateway for inference of large phylogenetic trees. In *2010 gateway computing environments workshop (GCE)* (pp. 1-8).

Murray F, Tarrant P. (2015). A social and economic impact assessment of cockle mortality in the Burry Inlet and Three Rivers cockle fisheries, South Wales, UK. Seafish, Welsh Government Marine and Fisheries Division. https://www.seafish.org/document/?id=18d2b595-0e32-4640-9030-eec0e2ec191f [Accessed May 2024]

Natural England. (2010). The Wash National Nature Reserve. NE273_The_Wash_NNR_Tagged.pdf (wwrg.org.uk). [Accessed November 2024]

Ramilo, A., Abollo, E., Villalba, A. and Carballal, M.J., (2018). A Minchinia mercenariae-like parasite infects cockles Cerastoderma edule in Galicia (NW Spain). *Journal of Fish Diseases*, *41*(1), pp.41-48.

Romalde JL, Vilariño ML, Beaz R, Rodríguez JM, Díaz S, Villalba A, Carballal MJ. (2007). Evidence of retroviral etiology for disseminated neoplasia in cockles (*Cerastoderma edule*). J. Invertebr. Pathol. 94(2): 95-101. https://doi.org/10.1016/j.jip.2006.09.007

Ronquist, F., Teslenko, M., Van Der Mark, P., Ayres, D.L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M.A. and Huelsenbeck, J.P., (2012). MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic biology*, *61*(3), pp.539-542.

Skujina I, Hooper C, Bass D, Feist SW, Bateman KS, Villalba A, Carballal MJ, Iglesias D, Cao A, Ward GM, Ryder DRG, Bignell JP, Kerr R, Ross S, Hazelgrove R, Macarie NA, Prentice M, King N, Thorpe J, Malham SK, McKeown NJ, Ironside JE. (2022). Discovery of the parasite *Marteilia cocosarum* sp. nov. in common cockle (*Cerastoderma edule)* fisheries in Wales, UK and its comparison with *Marteilia cochillia*. J. Invertebr. Pathol. 192: 107786.

Stillman RA, Moore JJ, Woolmer AP, Murphy MD, Walker P, Vanstaen KR, Palmer D, Sanderson WG. (2010). Assessing waterbird conservation objectives: an example for the Burry Inlet, UK. Biological Conservation 143: 2617 – 2630.

Tamura K, Stecher G, Kumar S. (2021). Molecular Evolutionary Genetics Analysis (MEGA) version 11. Molecular Biology and Evolution 30: 3022 – 3027.

Villalba A, Carballal MJ, López C. (2001). Disseminated neoplasia and large foci indicating heavy haemocytic infiltration in cockles *Cerastoderma edule* from Galicia (NW Spain). Dis Aquat Org 46(3): 213-216. https://doi.org/10.3354/dao046213

Villalba A, Iglesias D, Ramilo A, Darriba S, Parada JM, No E, Abollo E, Molares J, Carballal MJ. (2014). Cockle *Cerastoderma edue* fishery collapse in the Ría de Arousa (Galicia, NW Spain) associated with the protistan parasite *Marteilia cochillia.* Dis. Aquat. Org. 109: 55 – 80.

Ward GM, Bennett M, Bateman KS, Stentiford GD, Kerr R, Feist SW, Williams ST, Berney C, Bass D. (2016). A new phylogeny and environmental DNA insight into paramyxids: an increasingly important but enigmatic clade of protistan parasites of marine invertebrates. Int. J. Parasitol. 46(10): 605 – 619.

Ward, G.M., Feist, S.W., Noguera, P., Marcos-López, M., Ross, S., Green, M., Urrutia, A., Bignell, J.P. and Bass, D., (2019). Detection and characterisation of haplosporidian parasites of the blue mussel Mytilus edulis, including description of the novel parasite Minchinia mytili n. sp. *Diseases of Aquatic Organisms*, *133*(1), pp.57-68.Winnepenninckx et al. 1993

Woolmer A. (2013). Review of national cockle mortality issues and options for fishery management in Kent and Essex IFCA. Cockle Mortality and Biosecurity Management Review. Kent and Essex Inshore Fisheries

and Conservation Authority. https://cocklereview.kentandessex-ifca.gov.uk/sites/default/files/2022-04/Review%20of%20UK%20cockle%20fishery%20management%20.pdf [Accessed May 2024]