The susceptibility of Irish-grown and Galician-grown Manila clams, *Ruditapes philippinarum*, to *Vibrio tapetis* and Brown Ring Disease

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ABSTRACT

Brown Ring Disease (BRD), which affects the Manila clam in Europe, is caused by the bacterium, *Vibrio tapetis*. BRD has been diagnosed in Ireland on only one occasion (1997) although the aetiological agent has recently been detected in apparently healthy Manila clams from a number of sites around the Irish coast. The present work investigated the susceptibilities to BRD of two stocks of Manila clams, one from Ireland and the second from Galicia, north-western Spain, where BRD has been reported on a number of occasions. Exposure of the clams was by addition of *V. tapetis* to the holding waters. Development of BRD was assessed by the appearance of brown ring signs on the host shells, by bacterial isolation and characterization, and by detection of the bacterium by PCR. The pathogen was recovered from infected individuals and confirmed as *V. tapetis* by biochemical tests and a slide agglutination test. Galician clams experienced significantly higher mortalities, BRD prevalences and *V. tapetis* levels than Irish clams. Background infection with *V. tapetis* in the control stocks prevented conclusions being drawn on comparative susceptibility of the two stocks. Irish clams were significantly affected by the experimental challenge, as demonstrated by the development of BRD and an increase in *V. tapetis* levels. Results illustrate the vulnerability of Irish clams to BRD and have implications for the movement and transfer of clam seed in Ireland.

Key Words: *Vibrio tapetis*, Brown Ring Disease, Manila clam, Susceptibility, Ireland, Galicia

INTRODUCTION

The Manila clam, *Ruditapes philippinarum*, was introduced to Europe in 1972 (Flassch and Leborgne, 1992) and is now cultured for commercial purposes in a number of European countries. In 1987, the first outbreak of Brown Ring Disease (BRD) was reported from Brittany, France (Paillard et al., 1989). The disease is characterized by a brown conchiolin deposit, which forms a characteristic ring on the inner surface of the shell valves, and from which the disease is named (Paillard and Maes, 1990). The aetiological agent of BRD is the gram-negative bacterium, *Vibrio tapetis* (Paillard and Maes, 1990; Borrego et al., 1996). BRD has since been responsible for mortalities in the Manila clam from several European countries including France, Italy, Spain, Portugal, England and Ireland (Paillard et al., 1989; Paillard and Maes, 1990; Paillard et al., 1994; Figueras et al., 1996; Clarke, 1999; Allam et al., 2000).

BRD appeared in Spain in the summer of 1989 associated with mass mortalities of cultured Manila clams in the south-west, and later in Galicia in north-western Spain (Castro et al., 1992; Robledo et al., 1994; Castro et al., 1996; Figueras et al., 1996; Novoa et al., 1998). Although no BRD outbreaks have recently been reported in Galicia, results from a seasonal survey conducted within the last two years indicated a *V. tapetis* prevalence of approximately 50% in several clam populations from this area (Beaz et al., 2005).

69 In Ireland, BRD was reported from *R. philippinarum* from two areas in the north-west,
70 Donegal and Sligo, in 1997 (Clarke, 1999). *R. philippinarum* from Donegal had been on-
71 grown from seed imported from the U.K., which was suspected to be the source of BRD
72 infection, although this was never proven (Reid, *pers. comm*.). All stocks of the Manila
73 clam in Donegal were culled as a result and the former cultivation areas were
74 subsequently left fallow until 2002, when new Manila clam stocks, grown from native
75 seed, were planted. In Sligo, the early stages of BRD were diagnosed in 1997, but the
76 disease did not subsequently develop but rather disappeared soon after (Clarke, 1999).
77 BRD is not known to currently occur in Ireland. However, *V. tapetis* has recently been
78 detected in clams from a number of coastal bays in the north and west of Ireland
79 (Drummond, 2006; Drummond et al., *In Press*).
80
81 The presence of *V. tapetis* in Irish clams introduces the possibility of another BRD
82 outbreak, should other conditions e.g. environmental, immunological status of clams, be
83 suitable. Environmental factors are believed to influence clam susceptibility to BRD
84 (Paillard et al., 1997; Reid et al., 2003; Paillard et al., 2004), and in Ireland the annual
85 water temperatures in proximity to Manila clam cultivation beds range from 6ºC to 19°C
86 and annual salinities from 26ppt to 33ppt (Drummond et al., 2006), ranges known to be
87 favourable for the spread of BRD. Additionally, the increased importation of clam seed
88 for commercial on-growing purposes into Ireland raises the possibility of BRD
89 transmission from foreign seed. The purpose of the current infection trial was to analyse
90 the susceptibility to BRD of Irish-grown Manila clams in which BRD has never been
91 observed, in comparison to a Spanish stock of Manila clams from which BRD has been

observed in recent years, by exposing each stock to known concentrations of *V. tapetis* under controlled conditions. A further objective was to investigate *V. tapetis* prevalence in different host tissues.

MATERIALS AND METHODS

Clams

Healthy adult Manila clams of 35 to 40mm in length, aged 2+ years, and grown from Irish seed, were transported from Sligo, Ireland to Santiago de Compostela, Galicia. At the time of collection, clams were experiencing average daily temperatures of 12°C-14°C and salinities of 30ppt-33ppt. Additionally, adult Manila clams of 30 to 35mm in length and aged 2+ years were obtained from a depuration plant located in Ría de Villagarcía, Galicia, a region where BRD has previously been reported: clams had been experiencing average daily temperatures of 14°C and salinities of 32ppt-33ppt at the time of collection. A sub-sample of clams (n = 30) from each stock was analysed for brown ring signs in order to determine their BRD status. Clams from each stock were maintained separately in holding tanks containing water of temperature 16-17ºC and salinity of 36ppt for a period of one week in order to acclimatise.

Experimental Infection

For the experimental challenge, the *V. tapetis* strain, GR0202RD, was chosen. ~~There are believed to be a number of~~Two genetic ~~and serological~~and serological ~~-~~groups have been described ~~of~~ within *V. tapetis* strains isolated from clams (Rodríguez *et al.*, 2006):

isolates similar to the type *V. tapetis* strain B1090^T (Group 1) have ~~generally~~ been ~~characterised~~ associated ~~from~~ to the Manila clam, *R. phillipinarum* ~~(Paillard and Maes, 1990; Borrego et al., 1996; Novoa et al., 1998)~~, whereas isolates similar to strain GR0202RD (Group 2) are generally derived from the carpet-shell clam, *R. decussatus* ~~(Novoa et al., 1998)~~. ~~-~~In order to differentiate inoculated *V. tapetis* from the strain which might naturally occur in the experimental clams, strain GR0202RD was used for the experimental challenge. Although this strain is not known to occur naturally in *R. philippinarum*, it has been demonstrated to induce BRD in *R. philippinarum* at comparable rates and intensities as B1090^T (Romalde, unpubl. data).

Strain GR0202RD was plated to a marine agar (MA) plate and incubated for 48 hours at a temperature of 20ºC. Colonies were collected from the plate, suspended in marine broth and incubated at room temperature to reach an exponential growth phase. Liquid cultures were then centrifuged for 20 minutes at 10ºC and 5000rpm, and pellets were re-suspended in 50mls sterile seawater. The bacterial concentration was determined to be 5 x 10^8 cells ml^{-1} by the plate counting method on MA.

Two hundred Irish clams and two hundred Galician clams were placed in separate empty tanks, each forming a single non-overlapping layer of clams. 2L of sterile seawater were added to each tank, just enough to cover all clams. The bacterial suspension was added to the water of each tank, giving a final concentration of 6.25 x 10^6 cells ml^{-1}, and mixed to ensure even distribution of bacteria amongst the clams. Clams were left undisturbed for 3 hours. During this time, they were monitored in order to verify that their shells

were open and that they were actively filter-feeding, thereby facilitating the uptake of bacteria: in each tank, approximately 95% of clams were observed in this condition. The water was then drained and the clams remained out of water for 1 hour, in order to encourage closure of their valves and the subsequent incorporation of the bacterium within the pallial cavity. In separate control tanks, two hundred Irish control clams and two hundred Galician control clams were maintained and treated as above, except that the bacterial suspension was not added to the tanks.

Experimental Tanks

Eight 50L tanks were set up, consisting of two replicate tanks of the following four different experimental conditions: Irish clams exposed to bacterial suspension; control Irish clams not exposed to bacterial suspension; Galician clams exposed to bacterial suspension; and control Galician clams not exposed to bacterial suspension. Each tank contained 100 clams, at a density of 1 individual per 0.5L of seawater and at a temperature of 12±4ºC and salinity of 33±1ppt. Half of the volume of seawater in each tank was replaced with fresh seawater twice a week throughout the experiment. Clams were fed 6-8mls of a mixed diet of *Tetraselmis suecica* (3-6 million cells per ml) and *Isochrysis galbana* (30-40 million cells per ml) every third day throughout the trial, which ran for 52 days.

Sampling

Samples of four clams per tank were taken weekly for eight weeks, for detection of BRD on the interior shell valves. Of these, three clams per tank were used for detection of *V.*

161 *tapetis* by bacteriological techniques and by PCR. Three tissues from each clam, the
162 mantle, gills and extra-pallial fluid (EPF) were analysed. Tanks were checked on a daily
163 basis for mortalities and moribund clams. Any individuals which gaped were presumed
164 moribund and removed.
165
166 **Analysis of Brown Ring Disease**
167 Shells were retained and left to dry for macroscopic and microscopic detection of brown
168 ring signs on the inner surface of the valves. Disease progression on the shell valves was
169 monitored according to the classification system of Paillard and Maes (1994), in which
170 the syndrome is characterised by two stages which may occur simultaneously in a given
171 individual: a conchiolin deposit stage (CDS) and a shell repair stage (SRS). In the
172 current trial, only the CDS was utilised, as diseased animals did not exhibit evidence of
173 the shell repair stage. The determination of the CDS is based on an index which takes
174 into account the extent and thickness of the brown ring deposit. For example, CDS 1
175 (Stage 1) is not visible to the naked eye and can be seen only with a microscope. CDS 2
176 (Stage 2) corresponds to a limited deposition of conchiolin and CDS 3 through to CDS 7
177 to increasingly heavy deposition on the inner shell valves.
178
179 **Microbiological Isolation and Characterisation of *Vibrio tapetis***
180 The mantle and gills were dissected from every clam and each placed in 300µls of sterile
181 saline solution. Each tissue was homogenised by mashing with a plastic tip, and 100µls
182 of the solution was spread, using a glass spreader, on a Thiosulfate Citrate Bile Sucrose
183 (TCBS) agar plate. One hundred µls of EPF was extracted from each individual, by

184 inserting the syringe between the inner shell wall and the outer mantle edge, and spread
185 on a TCBS agar plate. Additionally, in the case of moribund and dead clams, a swab
186 from the soft parts was taken and plated onto a TCBS agar plate. All plates were
187 incubated for 24 hours at 20ºC.
188
189 From each TCBS plate, 1-2 green saccharose-negative colonies, i.e. suspected *Vibrio*
190 spp., were isolated on MA to obtain pure cultures, and subsequently stored in vials,
191 containing 15% glycerol and 85% marine broth at -80ºC, for further characterisation. All
192 the remaining colonies were collected from each TCBS plate, placed in eppendorf tubes
193 containing 1ml sterile distilled water and stored at -20ºC until further analysis by
194 Polymerase Chain Reaction (PCR).
195
196 A sub-sample of 40 isolates (6% of those available and representing clams in all tanks
197 and all weeks) were characterised by a series of standard morphological, physiological
198 and biochemical tests (Cowan, 1974; Smibert and Krieg, 1981; West and Colwell, 1984;
199 Borrego et al., 1996). In addition, they were assessed for growth in 0%, 0.5%, 3%, 6%
200 and 8% NaCl and at temperatures of 4°C, 37°C and 44°C. Sensitivity to the vibriostatic
201 agent O/129 was determined after 24 hours on Mueller-Hinton agar plates by using O/129
202 discs (150µg). Isolates which did not meet *V. tapetis* criteria were eliminated from
203 further successive tests, as results became available. Confirmative identification of
204 presumptive *V. tapetis* isolates was determined by serology: slide agglutination tests were
205 performed as described by Romalde et al. (1993) using anti-sera against *V. tapetis* strains,
206 $B1090^T$ and GR0202RD.

207

208 **Detection of *Vibrio tapetis* by PCR**

209 Bacterial colonies in suspension in sterile distilled water were thawed. DNA was
210 extracted using InstaGene™ Matrix (BIO-RAD) according to the manufacturers'
211 instructions and stored at -20ºC until required for PCR reactions. PCR amplification of
212 template DNA was undertaken using the primers and protocol developed by Rodríguez et
213 al. (2003; 2006): the primers can detect representatives of the three genetic groups
214 described for *V. tapetis*, including strains GR0202RD and $B1090^T$. One Pure Taq Ready-
215 To-Go PCR Bead (Amersham Biosciences) was used in every reaction: each bead
216 contained 1.5 units of Taq polymerase, 10mM Tris-HCl (pH 9.0), 50mM KCl, 1mM
217 $MgCl_2$, 200mN each dNTP. For a 25µl reaction, the bead was combined with 2pmol
218 (1µl) of forward primer, 2pmol (1µl) of reverse primer, 22µls of sterile distilled water
219 and 1µl of DNA template solution. The PCR products were electrophoresed on a 1%
220 agarose gel, stained with ethidium bromide and photographed under UV light. A 50 -
221 2,000 bp ladder (Sigma Chemical Company) was used as a molecular marker.

222

223 **Statistical analyses**

224 Significant differences in mortalities and in PCR-positive individuals amongst clam
225 groups were calculated by the Chi-square test, with the Yates' correction applied in cases
226 of two categories of data (one degree of freedom). Significant differences in BRD
227 prevalences amongst all clam groups were assessed by the Kruskal-Wallis test, followed
228 by the Mann-Whitney U-test for examination of pairwise differences.

229

RESULTS

Mortality

In total, there were 187 (23.4%) mortalities out of a possible 800 clams, of which 109 (13.6%) were derived from exposed tanks and the remaining 78 (9.8%) from control tanks. There was minor mortality (1.8%) in the first 5 weeks of the trial, with the majority (21.6%) occurring in the final three weeks. After 8 weeks, mean cumulative mortality reached 39.5% amongst Galician control clams and 51.4% amongst Galician exposed clams, whilst mean cumulative mortality for Irish control clams and Irish exposed clams was 11.9% and 20.4% respectively: the experimental exposure did not have a significant effect on mortality in either stock ($\chi^2_1 = 2.41$; $P > 0.05$ for Galician clams; $\chi^2_1 = 2.15$; $P > 0.05$ for Irish clams) (Figure 1). Significantly higher mortality was observed in Galician clams than in Irish clams exposed to the *V. tapetis* inoculum, ($\chi^2_1 = 16.53$; $P < 0.001$); and in Galician control clams than in Irish control clams ($\chi^2_1 = 15.89$; $P < 0.001$) (Figure 1). In effect, both the exposed and control Galician clams suffered higher mortalities than their counterparts in the Irish stock when held under the same experimental conditions.

A proportion of mortalities from all tanks, other than from Irish control tanks, exhibited the symptoms of BRD on their interior shell valves (Table 1). There was a statistically significant association between tank groups (source of clam, with or without

253 experimental *V. tapetis* exposure) and stage of BRD in mortalities ($\chi^2_9 = 24.76$, $P < 0.01$).
254 Both exposed and control Galician clams exhibited higher levels of CDS 1, CDS 2, CDS
255 3 and CDS 4 than their counterparts in the Irish stock.
256
257 **Macroscopic and Microscopic Analysis of BRD**
258 Amongst the four groups of (live) clams, there were significant differences in BRD
259 prevalences over the course of the trial (Kruskal-Wallis test, $P < 0.001$). Galician clams
260 exhibited 38% BRD prevalence at T_0. As the trial progressed, both exposed and control
261 Galician clams exhibited increased BRD prevalences, reaching 100% in exposed clams in
262 week 4 (Figure 2a). The experimental exposure to *V. tapetis* did not have a significant
263 effect on Galician clams (Mann-Whitney U-test, $P = 0.869$). At T_0, Irish clams showed
264 no clinical signs of BRD. Irish control clams did not develop the disease over the course
265 of the trial. The experimental exposure to *V. tapetis* had a significant effect on Irish
266 clams (Mann-Whitney U-test, $P = 0.014$), resulting in BRD prevalences of up to 50% in
267 weeks 5 and 6 (Figure 2a). There were significant differences in BRD prevalence
268 between Irish exposed clams and Galician exposed clams (Mann-Whitney U-test, $P =$
269 0.0002); and between Irish control clams and Galician control clams (Mann-Whitney U-
270 test, $P = 0.0002$).
271
272 The development of BRD was more advanced in both control and exposed Galician
273 clams than in Irish clams (Figure 2b). Irish control clams did not express BRD
274 symptoms, whilst Irish exposed clams exhibited CDS 1 and CDS 2 BRD. Amongst the

Galician clams, the exposed individuals showed greater BRD development than the control clams, with a number of exposed individuals displaying CDS 4.

PCR Detection and Microbiological Detection of *Vibrio tapetis*

V. tapetis PCR-positives were detected in a total of 31 individuals (16% of those analysed by PCR). In some individuals, the pathogen was detected in more than one tissue, so that a total of 43 PCR positives were detected. Of the 43 tissues positive for *V. tapetis*, 20 (46.5%) were detected in the mantle, 18 (41.9%) in the gills and 5 (11.6%) in the EPF (Table 2).

Low levels of background *V. tapetis* were detected in Irish control clams: the infection was not detected by PCR during the first three weeks of the trial, but subsequently reached detectable levels, perhaps encouraged by the unnatural holding conditions, during the latter half of the trial. After 8 weeks, the cumulative percentage of PCR-positive clams per (Irish control) tank reached a mean of 2.1% (Figure 3). Significantly higher *V. tapetis* levels were detected in Irish exposed clams (16.7%) ($\chi^2_1 = 6.84$; $P < 0.01$). In Galician control tanks, *V. tapetis* levels increased over the course of the trial attaining a cumulative mean of 12.5% PCR-positive clams per tank. The experimental challenge on Galician clams resulted in a significantly higher level of infection (33.3%) ($\chi^2_1 = 8.48$; $P < 0.01$). There were significantly higher PCR-positive detections in Galician exposed clams over Irish exposed clams ($\chi^2_1 = 5.42$; $P < 0.05$) and significantly higher detections in Galician control clams over Irish control clams ($\chi^2_1 = 4.34$; $P < 0.05$) (Figure 3).

Despite the background *V. tapetis* infection detected in control clams from both areas, no *V. tapetis* isolates were recovered from control clams during the experimental period. Three bacterial isolates (7.5% of those characterized) from clams exposed to the pathogen were biochemically and serologically characterized as *Vibrio tapetis* GR0202RD, the strain used in the inoculum. Two were derived from Galician exposed clams and one from an Irish exposed clam.

DISCUSSION

The prevalence and intensity of diseases of marine invertebrates are often affected by environmental factors (Chu and La Peyre, 1993; Motes and DePaola, 1996; Hauton et al., 2000; Reid et al., 2003; Paillard et al., 2004), as well as by host-related aspects such as inter-population variability and immune status (Cheng et al., 2004a and b; Huvet et al., 2004). The current study entailed two infection trials to evaluate the possible different susceptibilities to BRD of Manila clams of diverse geographic origin, Ireland and Galicia, under controlled conditions of temperature, salinity and exposure to *V. tapetis*. It has previously been demonstrated that French Manila clam populations are more susceptible to BRD, and are slower to recover from it, than populations from the USA (Allam et al., 2001).

This study demonstrated that both Irish and Galician Manila clam stocks were susceptible to BRD when infected by immersion in waters containing high concentrations of the pathogen. Background *V. tapetis* was detected in both stocks, although only the Galician-sourced clams displayed the clinical signs of BRD from the beginning, whilst Irish clams displayed none of the characteristic symptoms at T_0. [BRD is not a disease which is notifiable to the Office International Epizooties (OIE), and this could explain why BRD was prevalent in Galician clams used in the current study when there have not been recent reports of BRD in Galicia.] The inoculated *V. tapetis* strain, GR0202RD, was re-isolated from experimentally-challenged clams. Although no isolates were characterised from

344 control animals, Galician Manila clams have previously been shown to be infected with a
345 *V. tapetis* strain similar to the type strain, B1090^T (Romalde, unpubl. data), whilst a wild
346 isolate also similar to strain B1090^T, has recently been confirmed in *R. philippinarum*
347 from the Irish clam stock (Drummond, 2006). In addition, concurrent experiments
348 undertaken in the laboratory using the same Irish clam stock revealed that there was
349 background *V. tapetis* infection with a B1090^T-like strain (Drummond, 2006). *V. tapetis*
350 strains B1090^T and GR0202RD belong to different genetic and serological groups within
351 the species (Rodríguez et al., 2006), being therefore easily distinguishable. ~~B1090^T like~~
352 ~~strains have been associated with *R. philippinarum* from a number of European locations~~
353 ~~(Paillard and Maes, 1990; Borrego et al., 1996; Novoa et al., 1998); GR0202RD like~~
354 ~~strains have been detected only from *R. decussatus* (Novoa et al., 1998).~~ With detection
355 of background levels of the pathogen in both stocks, the subsequent effect of the
356 experimental infection with *V. tapetis* was the superimposition of an infection, of a
357 different strain, on top of the background infection already present.
358
359 Previous Manila clam – *Vibrio tapetis* infection trials experienced mortalities of 2%
360 (Reid et al., 2003) and 2% - 30% (Paillard et al., 2004) after 30 days of challenge. In the
361 current trial, mortality of clams reached an overall total of 23.4%, with most of these
362 mortalities (21.6%) occurring in the final three weeks. It appeared therefore that the
363 water quality and maintenance regime were satisfactory for the duration of the trial.
364
365 Both control and exposed Galician Manila clams experienced higher mortalities, BRD
366 prevalences and *V. tapetis* levels than their counterparts in the Irish stock. Stages of BRD

367 in dead clams also showed that both exposed and control Galician clams exhibited more
368 advanced BRD stages than their Irish counterparts, which possibly contributed to
369 mortalities. The experimental infection had less of an impact on the stocks than the
370 sources of the stocks, as the experimental exposure to *V. tapetis* did not result in
371 increased mortality in either stock. The experimental exposure had a significant impact
372 on the Irish stock in relation to BRD prevalence and *V. tapetis* levels. In contrast, the
373 experimental exposure of Galician *R. philippinarum* had a significant impact with respect
374 to *V. tapetis* levels, but not so for BRD prevalence and mortality: the challenge appeared
375 to be of less consequence to the Galician stock than its pre-existing infection and disease.
376 *V. tapetis* levels in Galician control clams increased over the course of the trial,
377 demonstrating the virulence of the background infection. The high BRD prevalences and
378 *V. tapetis* levels displayed by both exposed and control Galician clams appeared to be the
379 result of a latent infection present from their source, rather than the experimental
380 challenge. A threshold *V. tapetis* level of 10^3cfu ml^{-1} in the EPF is believed to be
381 required to induce BRD symptoms in *R. philippinarum*: the clam is likely to be
382 asymptomatic for BRD with a bacterial burden below this threshold level (Paillard *et al*.,
383 2004). Although no quantification of the *V. tapetis* levels were determined at T_0, it
384 appears that the Galician stock exceeded this threshold from the beginning of the
385 experiment.
386
387 The reasons why BRD occurs in Galician *R. philippinarum*, and not in Irish *R.*
388 *philippinarum* are not known. *V. tapetis* strain $B1090^T$ has been detected in both. It is
389 possible that environmental conditions are more conducive to the development of BRD in

390 Galicia, although average annual temperature and salinity patterns in both regions are
391 similar: temperatures of 6ºC to 19°C in Ireland and 11°C to 20°C in the Galician Rias,
392 and salinities of 26ppt to 33ppt in Ireland and 30ppt to 34ppt in Galicia (Drummond et
393 al., 2006; http://www.intecmar.org). Indeed, these temperature and salinity patterns are
394 generally characteristic of most BRD-affected regions (e.g. the Atlantic coast of Europe)
395 (Paillard, 2004), and so it may be that another environmental parameter e.g. dissolved
396 oxygen, turbidity, is influential in the development of BRD in the two regions.
397 Moreover, the intensive aquacultural activity (http://www.mexillondegalicia.org) and an
398 environmental carrying capacity possibly approaching its limit in the Ría de Villagarcía,
399 as compared to the north-west of Ireland, may be conducive to development of the
400 disease: the associated conditions of reduced water quality and proliferation of pathogens
401 may lead to more stressful conditions for Galician clams. It has previously been
402 suggested that Galician *R. philippinarum* are already weakened by a high prevalence of
403 potential pathogens such as haplosporidians and *Perkinsus*-like organisms, and that these
404 pathogens act synergistically with *V. tapetis*, thereby increasing clam susceptibility to
405 BRD in Galicia (Figueras et al., 1992; Figueras et al., 1996). In contrast, the stock of
406 Irish-grown Manila clams used in this trial was free of *Perkinsus* sp. and has low
407 prevalence of other parasites, such as trematodes and turbellarians (Drummond, 2006).
408 Nevertheless, the results have shown that Irish *R. philippinarum* are susceptible to BRD
409 caused by *V. tapetis* strain GR0202RD: this strain has not been recorded in Irish clams to
410 date.
411

412 It has been reported that the major site of infection in BRD-diseased clams is the
413 periostracal lamina, and that *V. tapetis* is most abundant in the EPF (which is in contact
414 with the periostracal lamina) following BRD infection (Paillard and Maes, 1995a and b;
415 Allam et al., 1996). In the present study, PCR analysis was the sole method employed for
416 assessment of the presence of *V. tapetis* in a particular tissue. The pathogen was detected
417 more frequently on the mantle and gills than in the EPF, suggesting that the former
418 tissues provide the maximum chance of *V. tapetis* detection, at least for clams of 30-
419 40mm length. Concentration of *V. tapetis* on the gills is likely to have occurred during
420 the clam's filter-feeding process. Allam et al. (1996) reported that the mantle is not
421 generally deeply colonized, as the pathogen is not detected here following rinsing with
422 alcohol. In our study, the mantle was not rinsed with alcohol, suggesting that the bacteria
423 may have been concentrated on the surface of the mantle, rather than deeper within the
424 mantle tissue.
425
426 Results obtained clearly show that Irish clams were significantly affected by the
427 experimental challenge in terms of BRD prevalence and *V. tapetis* levels. The
428 vulnerability of Irish stocks of Manila clams to *V. tapetis* and BRD is demonstrated and
429 consequently has implications for clam movements: it is recommended that importation
430 of clam seed, of both *R. philippinarum* and *R. decussatus*, into Ireland from *V.*
431 *tapetis*/BRD-affected regions be discouraged. Further studies are needed to determine
432 the existence in Manila clams of inter-population variabilities in terms of BRD
433 susceptibility.
434

435

436

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438

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593 Table 1: *Ruditapes philippinarum* and *Vibrio tapetis*. Percentages of Manila clam
594 mortalities displaying stages of Brown Ring Disease on the shell valves. n = 187
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	Irish Exposed n = 31	**Galician Exposed n = 78**	**Irish Control n = 18**	**Galician Control n = 60**
% Negative for BRD	64.5	46.2	100	38.3
% CDS 1	9.7	16.7	0	15.0
% CDS 2	19.4	25.6	0	31.7
% CDS 3	6.4	6.4	0	10.0
% CDS 4	0	5.1	0	5

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607 Table 2: *Ruditapes philippinarum* and *Vibrio tapetis*. Distribution of *V. tapetis* PCR-
608 positives detected in three tissues of the Manila clam. Numbers represent the total
609 detected amongst two replicate tanks of each treatment, from which three clams per tank
610 were sampled on a weekly basis. Clams, n = 192: PCR assessments, n = 576.
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PCR-positives	**Mantle Only n = 11**	**Gills Only n = 9**	**EPF Only n = 0**	**Mantle & Gills n = 6**	**Mantle & EPF n = 2**	**Gills & EPF n = 2**	**Mantle & Gills & EPF n = 1**	**Total n = 31**
Mantle	11	-	-	6	2	-	1	**20**
Gills	-	9	-	6	-	2	1	**18**
EPF	-	-	0	-	2	2	1	**5**
Distribution amongst tissues	11	9	0	12	4	4	3	**43**

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FIGURE LEGENDS

Figure 1: *Ruditapes philippinarum* and *Vibrio tapetis*. Percentage cumulative mortality of Irish-grown and Galician-grown Manila clams. Numbers of mortalities were averaged from two replicate tanks. n = 800: 400 exposed clams and 400 control clams.

Figure 2: *Ruditapes philippinarum* and *Vibrio tapetis.* (a) Prevalence of BRD in Irish and Galician clams experimentally exposed to *V. tapetis* and (b) Development of BRD in *V. tapetis*-exposed Irish and Galician clams. Weekly samples of four clams per tank were taken, and results averaged over two replicate tanks. n = 256.

Figure 3: *Ruditapes philippinarum* and *Vibrio tapetis*. Mean cumulative percentage of *V. tapetis* PCR-positive clams per tank. Three clams were sampled from each tank on a weekly basis. Three tissues, the mantle, gills and EPF, from each individual were analysed: a clam in which a PCR positive was detected was counted as one, even if more than one tissue in that individual was PCR positive. n = 192.

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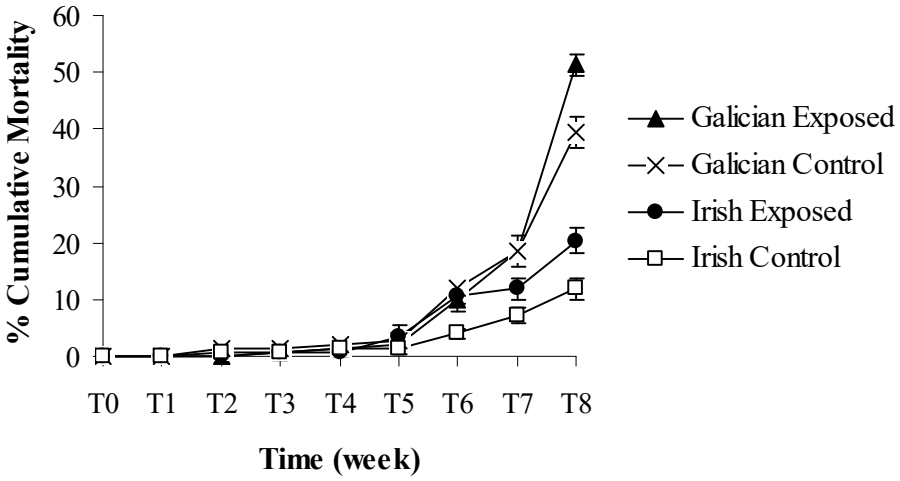

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651 Figure 1: Drummond et al.

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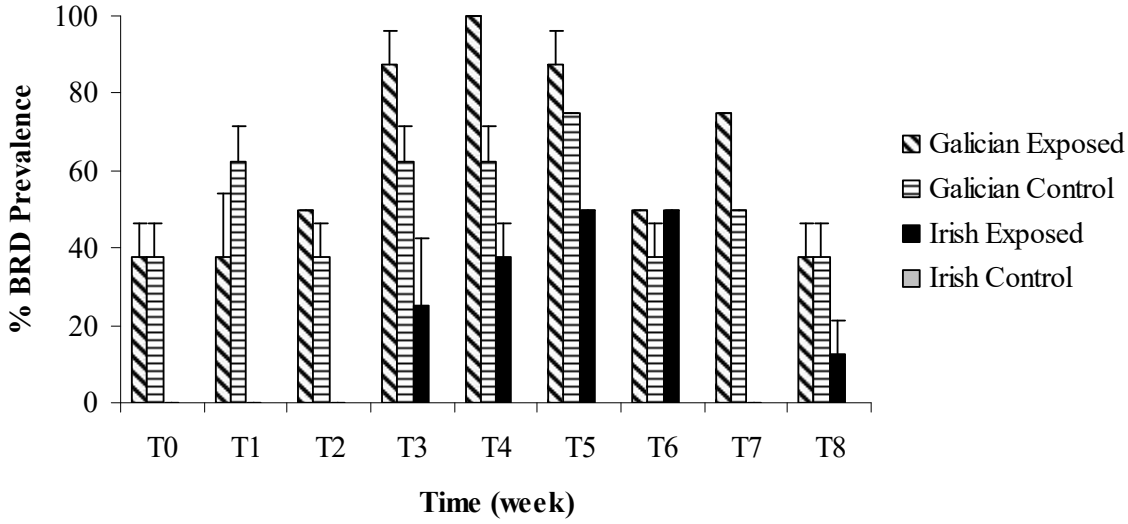

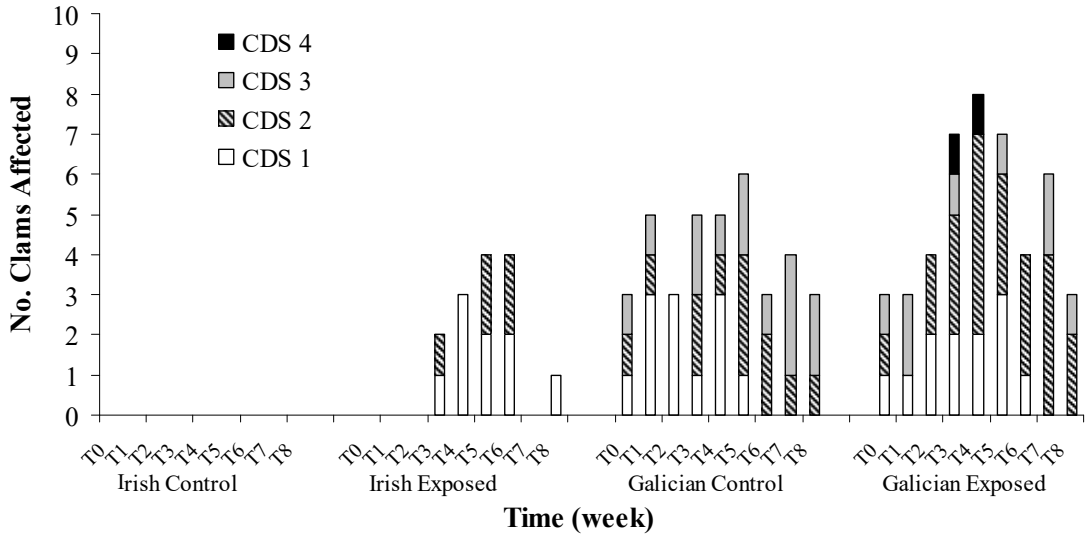

Figure 2: Drummond et al.

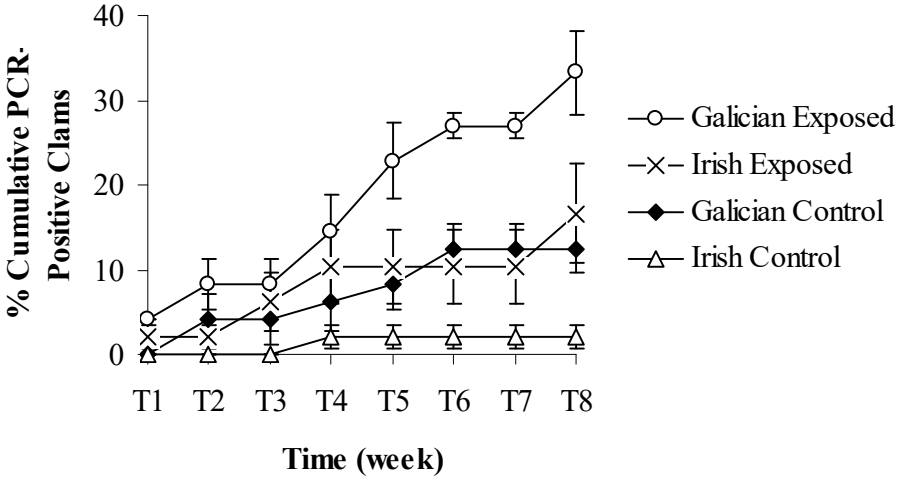

Figure 3: Drummond et al.