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Chapter

AN ECOLOGICAL APPROACH TO UNDERSTANDING HOST-PATHOGEN-ENVIRONMENT INTERACTIONS: THE CASE OF BROWN RING DISEASE IN CLAMS

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ABSTRACT

Brown Ring Disease (BRD) is used as a case study to describe an ecological approach to understanding host-pathogen interactions. BRD is caused by a bacterial microparasite, *Vibrio tapetis*, which elicits the inhibition of the calcification process. Epidemiological studies of BRD are facilitated by the use of characteristic clinic signs on the inner surface of the valves. The phenology and dynamics of BRD are then described by coupling epidemiological, ecological and physiological data. Spatial distribution of BRD prevalence in natural clam populations and *V. tapetis* load in the sediment allows the investigation of the transmission mode of this pathogen. Experimental testing of different transmission modes has demonstrated the predominant role of feces and pseudofeces in *V. tapetis* transmission and diffusion processes. Some other hosts can harbor *V. tapetis*, including reservoir species such as cockles but also vertebrate predators such as fishes, and cross-species transmission could potentially play a major role in dispersion of the pathogen during its life cycle. Environmental factors such as temperature, salinity and nutrients strongly modulate the coevolution between *Vibrio* and bivalve.

Keywords: microparasite, *Vibrio*, bivalve, transmission, clinical signs, shell disease, environment, temperature

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INTRODUCTION

Taking an ecological approach to the investigation of host-pathogen interactions primarily requires a natural environment approach known as epidemiology. Investigating the distribution of the disease in space and time allows analysis of the factors (environmental, genetic...) concurrent with its onset and development. In combination with laboratory experiments, this approach can be used to assess the role of key factors on host-pathogen interactions. The development of a numerical model of BRD allows knowledge to be synthesized in a simple manner (etiology, diagnostic tools, pathogen characterization, mechanisms of host-pathogen interaction, epidemiology), to prioritize different stages and to point to gaps and flaws of the three component system, Environment, Host and Pathogen (HPE) [22]. The HPE system can be reconsidered taking into account the entire microbial community: on one hand, the pathogen community and the co-infection processes, and on the other hand, the microbiota that the host harbors [9, 27, 28]. This ecological approach is highly interdisciplinary, drawing on techniques from fields including genetics, molecular biology, genomics, immunology, epidemiology and modeling (Figure 1).

EPIDEMIOLOGICAL APPROACH

Epidemiology is the study of diseases and health factors (environmental, genetic) in a population. The originality of epidemiology is its focus on the population rather than the individual. Quantification is paramount in epidemiology and requires to be precise and rigorous to determine the epidemiological indicators for each type of disease. These indicators will most often be expressed in relative terms compared to the population in which it is observed.

Brown Ring Disease (BRD) provides a useful case study to describe the uses of epidemiological parameters, because the disease diagnostic is based mostly on macroscopic signs. In fact, BRD is due to a bacterial microparasite, *Vibrio tapetis*, which elicits shell growth abnormalities by inhibiting calcification process and inducing the deposit of brown conchiolin between the mantle and the inner surface of the valves [20]. This defense process, known as nacrezation or pearl formation [5] embeds parasites inside the shell matrix. This shell repair process is very powerful against macroparasites, such as trematodes, but in the case of microparasites such as bacteria with high generation rates, this mechanism is less efficient. However, depending on the HPE interaction modulation factors, it has been demonstrated that conditions favorable to the host such as nutrition and high temperature could enhance BRD recovery [13, 19, 23].

The development of clinical signs for BRD using a classification system to assess conchiolin deposition, shell repair and phase of the disease has already been established (Figure 2) [18, 20].

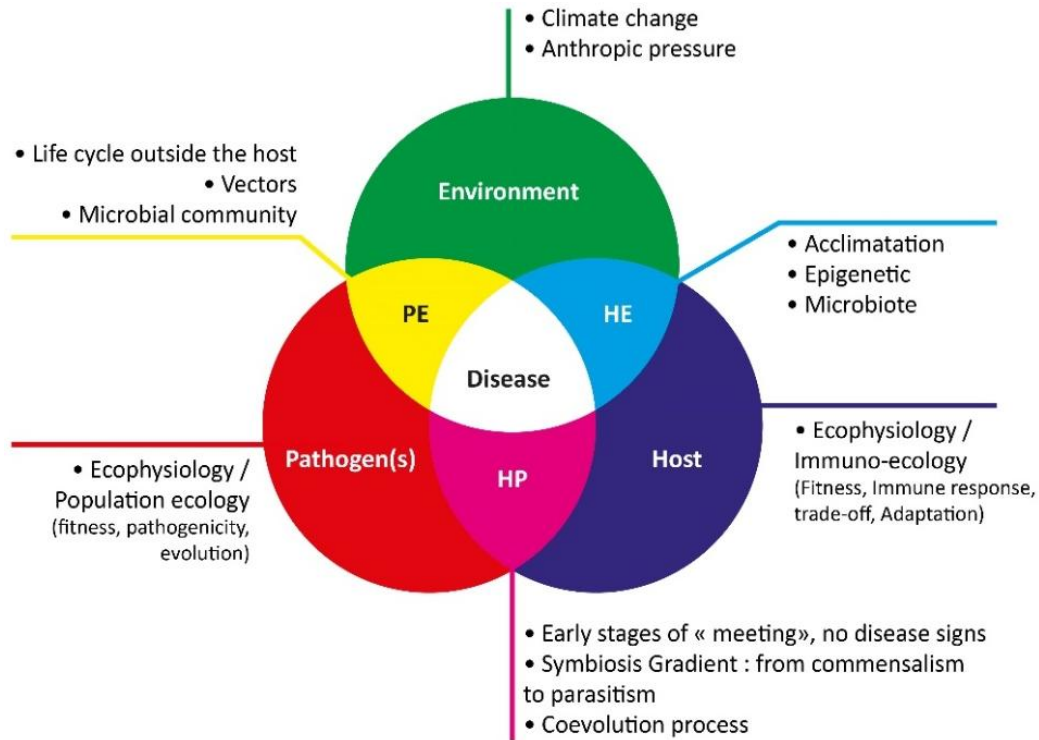


Figure 1. The ecological approach of the Host-Pathogen-Environment System.

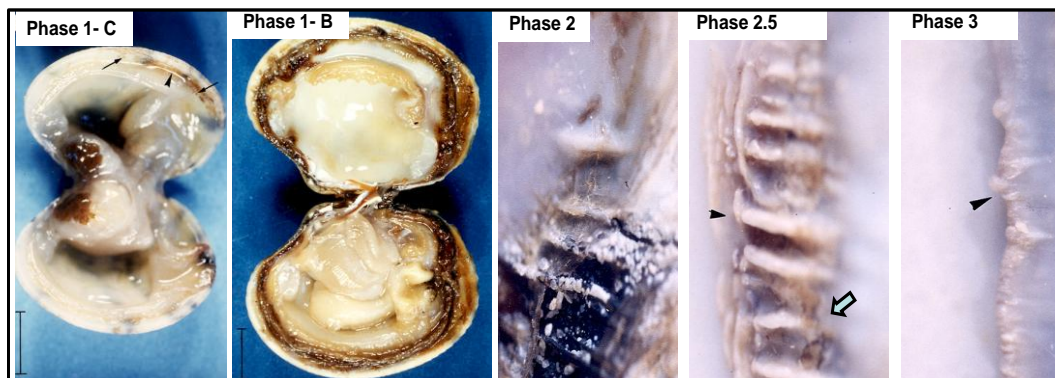


Figure 2. BRD clinical signs (BRD+) that could be observed macroscopically and microscopically from Phase 1 to Phase 3 of Brown Ring Disease. In Phase 1 (cases B and C), only conchiolin deposit is observed without shell repair. In the other phases (Phase 2, 2.5 and 3), the conchiolin deposit is recovered by shell repaired layers at different recovery steps, intermediate (phase 2), almost recovery (Phase 2.5) and completely recovery (Phase 3).

Epidemiological Indicators Can Be Used to Describe and Quantify Brown Ring Disease (BRD)

Prevalence of BRD (BRD%): total number of individuals infected with BRD clinical signs as a percentage of the total number of sampled individuals ($n \geq 100$), at a given moment. The prevalence provides static information about the frequency of the disease, but not its development (increase, decrease or increase of new cases, such as BRD intensity). It corresponds to the balance of a disease at a given time. The prevalence is not to be confused with the incidence, which is the rate at which newly infected BRD individuals appear in a specific population during a given period.

Prevalence of conchiolin disease stages (CDS%): number of individuals with a specific stage of brown ring relative to the total number of sick animals, during a specific time period or at a given time.

Prevalence of shell repair stages (SRS%): The number of individuals at shell specific stage of repair as a percentage of the total number of animals either currently sick or previously sick (and now recovered) during a specific time period or at a given time.

Another type of indicator is the pathogen load (number of cells or CFU/ml) in clams or other hosts or in the surrounding environment (sediment, seawater). Pathogen concentration is measured using different techniques previously described (bacteriological analysis, ELISA or SSP-PCR, qPCR) [22, Bidault et al., 2015].

Considering all epidemiological indicators together, BRD in clams can be considered in a phenological way, taking into account not only the changes in the clinical signs, but also the pathogen load and mortality of clams that is not necessarily related to the presence of BRD signs. Figure 3 summarizes these results and integrates them to form a more complete vision of the vibriosis, taking into account changes in pathogen load and BRD microscopic clinical signs.

Six Phases of Vibriosis in Clams Can Be Identified Over Time

Six phases of vibriosis in clams can be identified over time.

- Phase 0 is characterized by the absence of both BRD clinical signs (BRD-) and the pathogen after detection. It therefore represents either a healthy individual, or a clam beginning BRD incubation, with a pathogen load below the limit detection of *V. tapetis* by qPCR (1.125×10^1 bacteria/ml)[Bidault et al., 2015]
- Phase 0.5 is characterized by an absence of BRD clinical signs (BRD-), but by obligatory positive detection *V. tapetis*. *V. tapetis* load can be measured in the extrapallial fluids (Figure 3, cases B or C), and/or in the hemolymph and tissues (Figure 3, cases A or B). It is a phase characterized by an absence of external BRD clinical signs and the presence of internal alterations such as increased hemocyte concentration a few hours after the inoculation, both in the mantle cavity and in the adductor muscle [3]. For clams which develop shell repair defense mechanisms, this phase is only temporary. For the others (cases A and B), invasion of *V. tapetis* within tissues will lead to mortality.

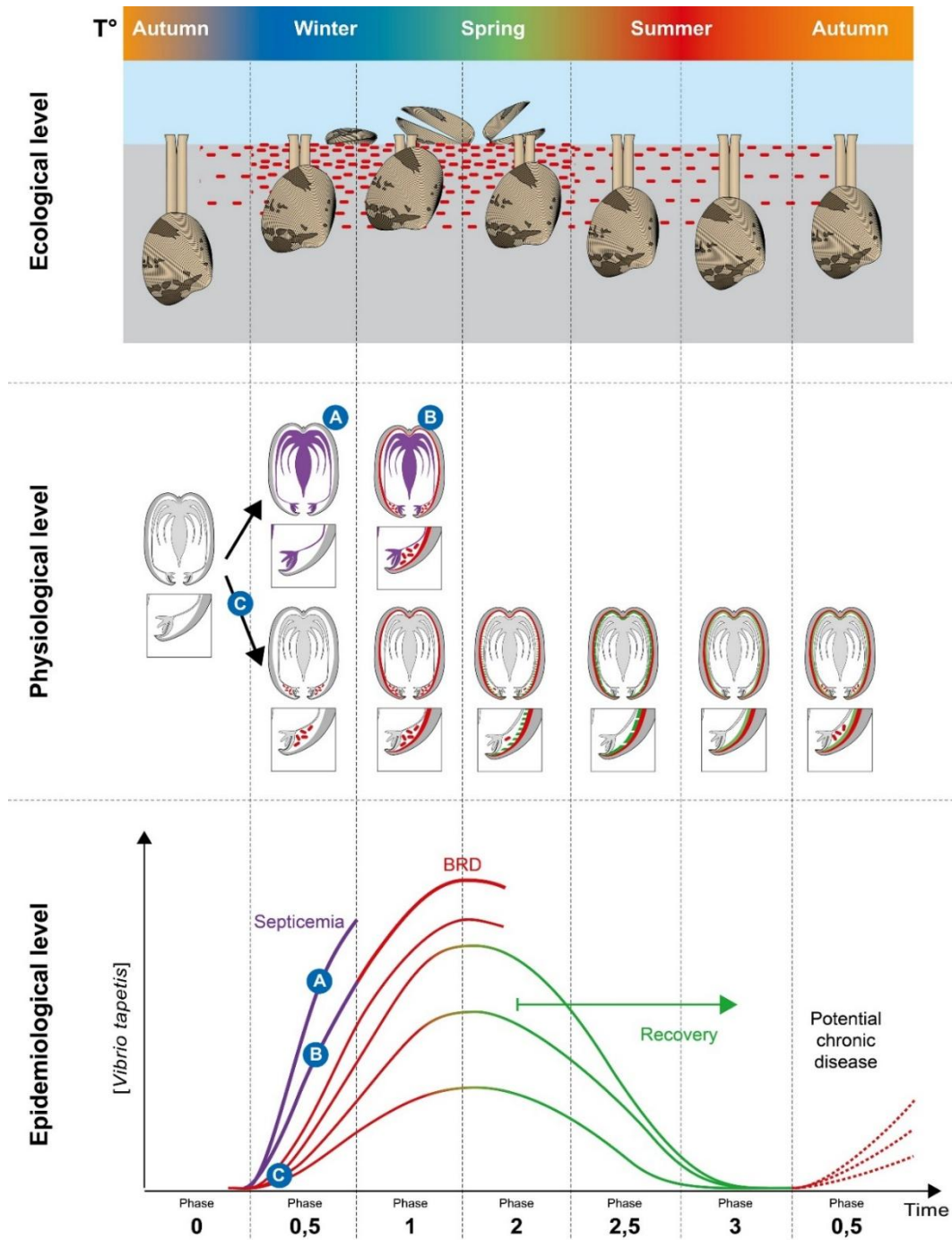


Figure 3. Synthesis of Brown Ring Disease (BRD) phenology, described at ecological, physiological and epidemiological levels. A: *Vibrio tapetis* only within tissues (purple); C: *V. tapetis* only in extrapallial and pallial fluids (red); B: *V. tapetis* in internal and external compartments (purple and red). Recovery process in green.

Phases 1 to 3 are all characterized by clinical signs (Figure 2).

- Phase 1 consists of the appearance and development of the conchiolin deposit (CD). *V. tapetis* concentration in extrapallial fluids tends to increase with CD stages.
- Phase 2 or the intermediate repair phase of BRD. The concentration of *V. tapetis* reaches maximum values.
- Phase 2.5 or the healing phase or "recovery". The concentration of *V. tapetis* starts to decrease slightly, reaching values similar to beginning of Phase 1. If there is no relapse, recovery will occur soon after.
- Phase 3 characterizes a recovered animal. A scar is visible on the shell and the concentration of *V. tapetis* is below the limit detection of *V. tapetis* [22]. The absence of *V. tapetis* must be confirmed using qPCR method [Bidault et al., 2015] during this phase.

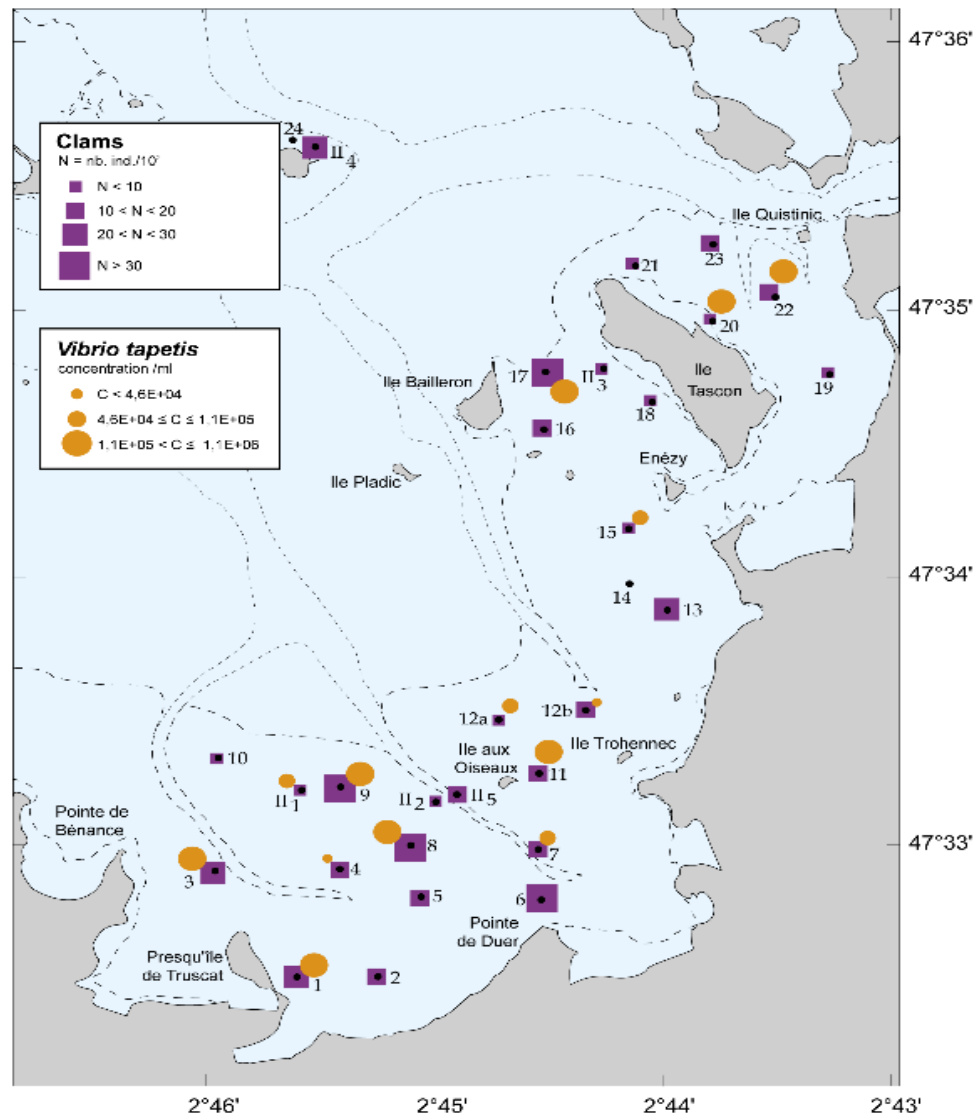


Figure 4a. Density of Manila clams (purple squares) and *V. tapetis* concentrations in the surface (0-2 cm) sediment (yellow circles) in the Gulf of Morbihan, France. March campaign 1999.

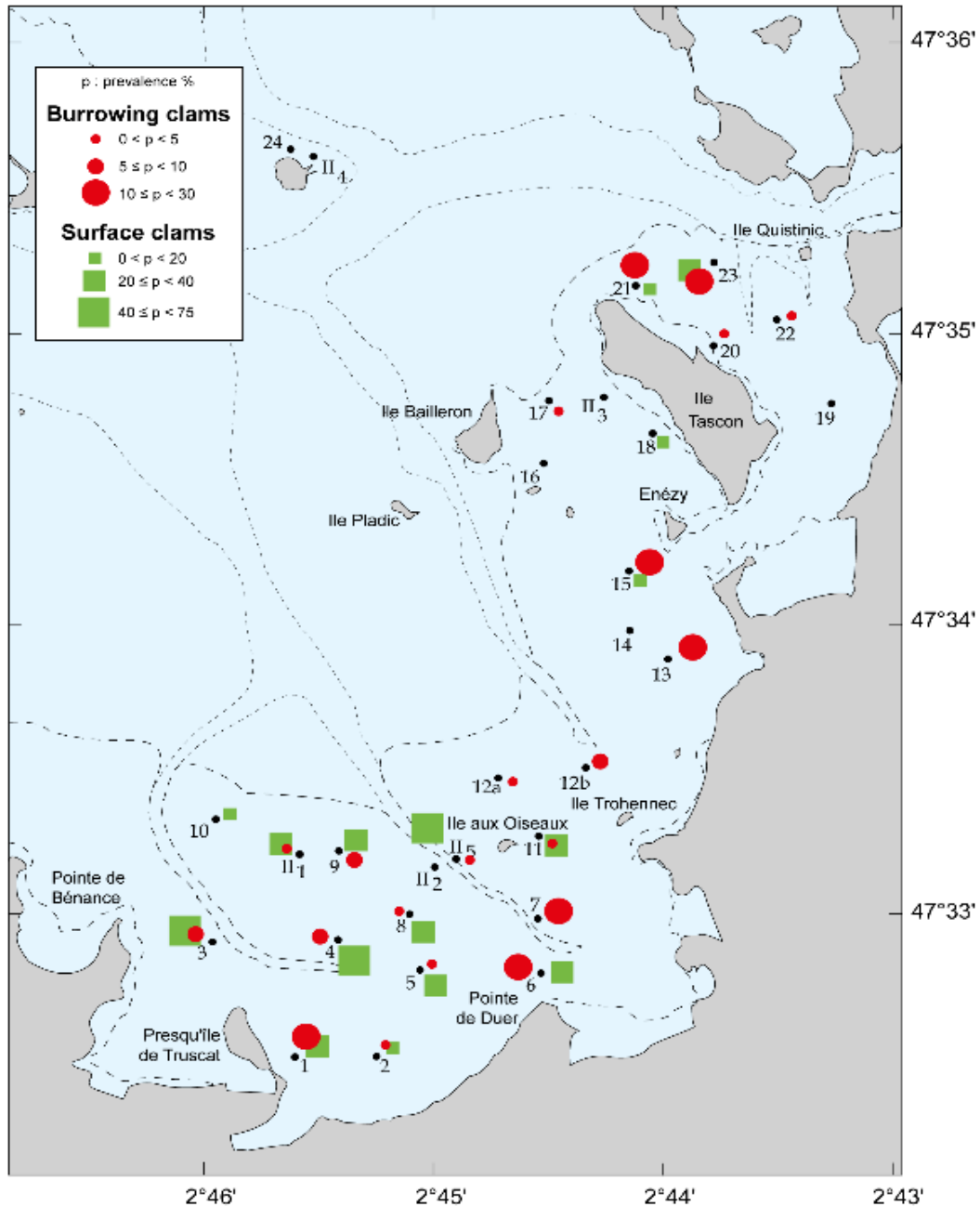


Figure 4b. BRD prevalence in burrowing (red circles) and in rising clams at the surface of the sediment (green squares) in the Gulf of Morbihan, France. March campaign 1999.

BRD associated mortalities occur generally in clams in phase 0.5 (cases A and B) when *V. tapetis* has colonized tissues, but also in phase 1 and 2, when the clams exhibit strong development of brown ring disease (phase 1 case B) or when the shell repair process is not completely finished (Phase 2, cases B and C) (Figure 2).

Using these indicators, Figure 3 represents a synthesis of BRD dynamics coupling prevalence of disease phase and *V. tapetis* load at individual level. It is interesting to note that mortalities can also occur without BRD signs (if the bacteria is able to penetrate within tissues and multiply in the hemolymph) [3] and at later stages of BRD development (in advanced stages of phase 2) [22].

The prevalence of BRD phase could represent a pertinent integrative indicator to determine health status of clam population.

Spatial Distribution of BRD and *V. tapetis* in the Field

Spatial distribution of disease (prevalence, pathogen load...) is also very useful in epidemiological studies. Spatial distribution of BRD prevalence was assessed according to the host density and density of pathogens in sediment (performed by ELISA) [17] during field sampling carried out during spring in the Gulf of Morbihan (Figures 4a and 4b). The distribution of BRD prevalence among burrowing and rising clams did not show any relationship with either host densities or pathogen densities in clams and in the sediment. However, a significant relationship between the density of the clams and the average concentration of *V. tapetis* in the sediment was demonstrated. Areas with very high densities of clams were also those that harbored the highest densities of *V. tapetis* in sediments. Simulations of Lagrangian trajectories for different numbers of particles or "larvae" using the hydrodynamic model of the Gulf of Morbihan [12] have confirmed that the areas with high densities of clams in the center of Truscat sandbank (cf. Figure 4a, purple squares) were those subject to low current swirling waters, favoring the accumulation and deposition of fine particles [10]. Larval transport and the spread of bacteria could also follow the same pattern of dispersion within the Truscat sandbank, resulting in the strong correlation between clam density and pathogen load.

DISEASE TRANSMISSION MODES

Considering the results of spatial distribution of BRD and *V. tapetis* in the natural population of clams, an experiment was performed to investigate transmission of BRD. Three disease transmission modes were considered: by water, via contact, and by feces and pseudofeces. To determine the role of water in transmission, the animals were placed just under the surface, on top of a grid that allowed their feces to sink to the bottom of the tanks. Clams were not in contact with feces or with other clams. For testing the role of contact transmission, the bivalves were placed in the same conditions as above; however, they were kept in continuous contact with each other. To simulate the process of infection by feces and pseudo-feces, clams were fixed to the bottom of the tank, without contact with each other. Feces and pseudo-feces were resuspended by stirring every two days. *V. tapetis* contamination was performed by placing *V. tapetis* inoculated individuals (10% of the total number of clams) in each aquarium. Each transmission mode was investigated at two different densities: 100 and 400 clams/m². For each transmission mode and for each clam density, the effect of water change was also tested (no water change, or water change every

two days). For this experiment, because disease-free clams were not available in the wild or from hatcheries, a treatment bath of chloramphenicol (8 mg/l for 48 hours) was performed on the clams prior to the start of the experiment. A subsequent thermic treatment (clams were subjected to a temperature change of 4°C every 24 hours, starting at the experimental temperature of 15°C, rising to a maximum of 27°C, and falling again to 15°C) and another antibiotic bath of 48 hours were applied to limit the development of opportunistic bacteria. The animals were fed every two days. After one month, the animals were sacrificed and the pathogenic load in the pallial and extrapallial fluids of clams determined by the ELISA method [17]. The results are summarized in Figure 5.

Whether water was renewed or not, the concentration of *V. tapetis* in fluids of clams placed in tank bottom in contact with feces and pseudofeces was significantly higher than those placed in open water or in intimate contact between each other. We were able to demonstrate that the concentration of *V. tapetis* in diseased clam feces represented 80 to 90% of the total heterotrophic microflora. When clams were at high density, water exchange allowed a reduction in the pathogen load (factor 10). Transmission through the water only appeared to be effective if the density of the host was high (at least 400 clams/m²) and if the water was not renewed. These are the conditions under which clams live in the Gulf of Morbihan (muddy sediments and densities of 100-600 clams/m²) (Figure 4a). However, within the trays on which the animals were placed in intimate contact, the distribution of hosts with high pathogen loads was independent of the location of the animals inoculated with *V. tapetis*. In conclusion, two possible transmission paths have been demonstrated: by water and by feces. Whatever the density of clams or water renewal treatment, transmission through the feces seems crucial.

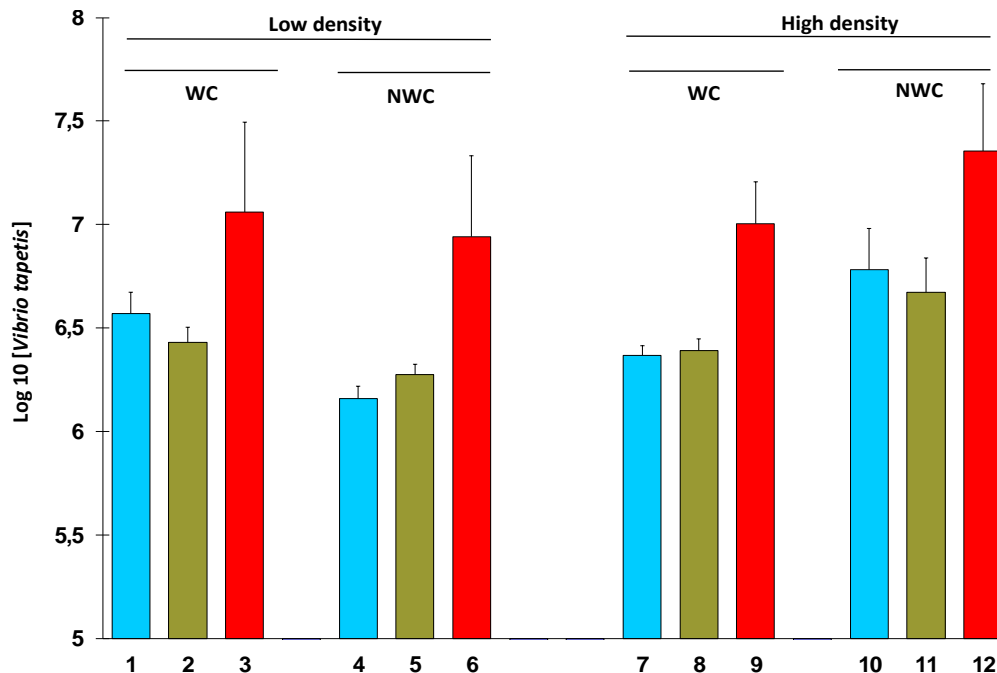


Figure 5. Log₁₀ [*V. tapetis*] in the pallial and extrapallial fluids of clams for each density condition and transmission mode at day 30. Blue: transmission by water; green: contact transmission; red:

transmission through feces. WC: with water change every two days. NWC: No water change. Low density (100 clams/m²); High density (400 clams/m²).

Vertical transmission of *V. tapetis* was also considered, but to date, no experiments have been conducted to test this hypothesis.

LIFE CYCLE OF *V. TAPETIS*

Previously, we have shown that *V. tapetis* could be found in sediment, where high clam density occurs. Rodrigues et al. [26] have demonstrated the ability of *V. tapetis* to form a biofilm *in vitro* on abiotic surfaces. Within the clams, a biofilm of *V. tapetis* on shell secretions constitutes the first steps of the infectious process. Then, *V. tapetis* multiplies in different compartments, primarily in the brown deposit, the periostracal secretion, the external mantle epithelium and the pallial and extrapallial fluids [1,2]. In times of winter mortality or pre-spring time, *V. tapetis* can be also isolated in the hemolymph of moribund clams [Paillard et al., unpublished data]. *V. tapetis* can also be detected during the same period in the pore water of the sediment, the water surrounding moribund clams, and in the feces and pseudofeces of BRD clams after placing them in sterile vials [16].

Hosts other than *R. decussatus* and *R. philippinarum*, have also been identified. *V. tapetis* has been isolated from other species of veneridae that suffer from BRD (*Venerupis aurea*, *Tapes rhomboïdes*, *Dosynia exoleta*), and the cockle, *Cerastoderma edule*, has also been identified as a potential vector species [15]. Indeed the ability to cross species barriers is a feature of *V. tapetis*, and can also occur from invertebrates to vertebrates. In fact, *V. tapetis* has been isolated from several species of fishes during their mortality events, such as flat fishes (*Hypoglossus hypoglossus*, *Dicologlossa cuneata*, *Solea solea*) and the wrasse (*Symphodus melops*) [7, 11, 14]. All these fishes are likely to be predators of clams and could also potentially be considered as secondary hosts of *V. tapetis*. Figure 6 summarizes the life cycle of *V. tapetis* as a pathogen of clams.

VIBRIO BEHAVIOR MANIPULATION?

The two schematic figures presented (ecological level of Figure 3 and Figure 6) summarize our knowledge of how micro-habitats, transmission modes and seasonal change are able to influence the spread of *V. tapetis*. It thus appears that a predation/parasitism interaction could exacerbate mortalities; as weakened infected bivalves rise to the surface of the sediment and are more accessible to predators. In addition, the rising clams have a very high pathogenic load of vibrios, varying from 2×10^5 to 8×10^9 CFU/ml, which is on average 100 times higher than in a burrowing clam. The rising clams therefore also constitute a source of contamination and spread of the pathogen. Could this be similar to the favourisation process induced by trematode parasites in the clam *Tapes aureus*? [6]. These parasites modify the host behavior by promoting its rising in the sediment, making it more accessible to the secondary hosts (birds) [4]. Indeed, this same behavior was observed for heavily *V. tapetis* infected clams, and rising clams could even act like delayed explosive bombs in the predator!

Thus, fish-eating clams, such as wrasse and flatfish may play a major role in the infectious cycle of *V. tapetis*, especially during periods of winter mortality.

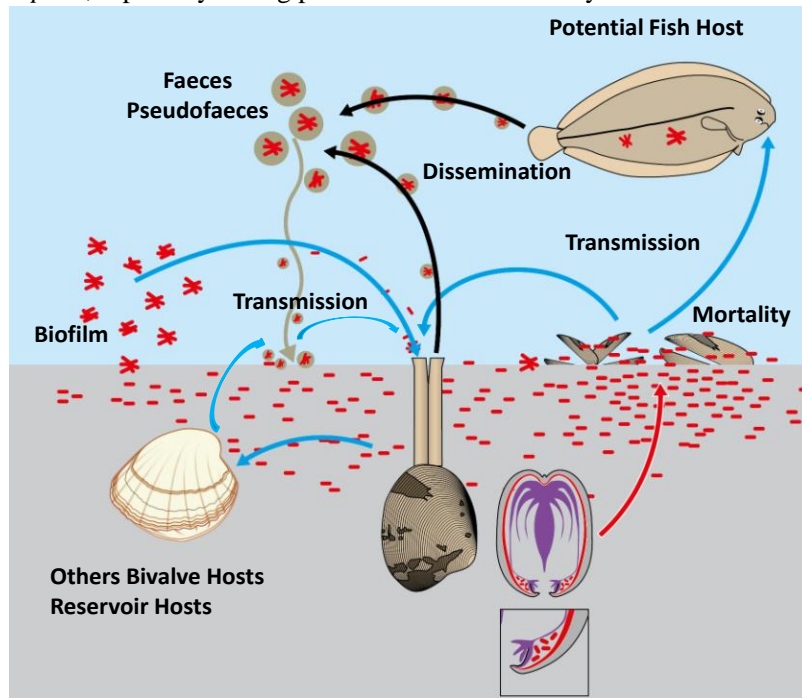


Figure 6. Life cycle of *V. tapetis* pathogenic to clams. The black arrows indicate dissemination, the blue arrows indicate transmission modes and the red arrows indicate mortality associated with the rising of the clams to the surface of the sediment.

IMPACT OF ENVIRONMENTAL FACTORS ON THE HOST-PATHOGEN INTERACTIONS

In natural populations of Manila clam along the coasts of Brittany (Bay of Brest, North Finistere and Morbihan), we have shown previously that maximum prevalence occurs during the winter and beginning of spring periods, peaking in March. Animals from one natural population in the Gulf of Morbihan, (“Île aux oiseaux”) were sampled during this spring period and maintained in aquaria at different temperature (4, 14, 18 and 21°C), allowing quantification of the BRD dynamic. At 14°C, the prevalence reached 90% after two months (Figure 7). At other temperatures (4, 18 and 21°C), the clams developed BRD but in much lower proportions. Environmental conditions strongly modulate the development of BRD. Experimental infections in laboratory with *V. tapetis* at various temperature have also shown that maximum BRD development in clams occurs at 14°C, and that at 21°C the prevalence is very low due to high recovery rates.

Temperature is also a major modulating factor impacting growth and survival of *V. tapetis*. Maximum growth occurs around 22°C but its survival decreases rapidly after 25°C and death is observed at 27°C [21]. The death of *V. tapetis* at a temperature generally measured in the sediment at summer conditions could explain the low prevalence of BRD in southern sites such as the Adriatic and Mediterranean Seas.

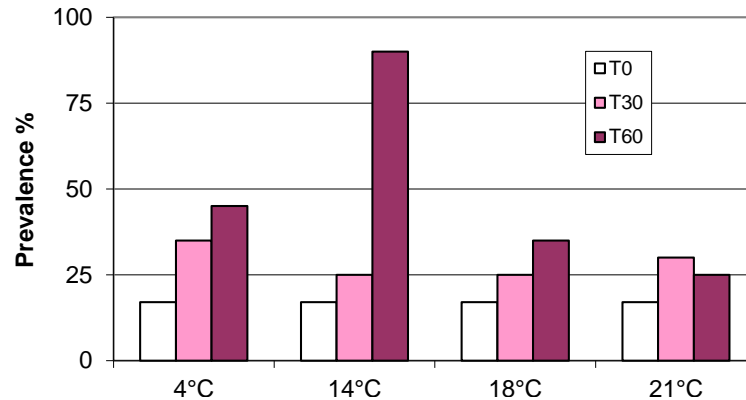


Figure 7. Evolution of BRD prevalence in clams harvested from natural populations (Île aux oiseaux, Gulf of Morbihan) in March 1998 and maintained in aquariums without food for two months at different temperatures (4, 14, 18 and 21°C).

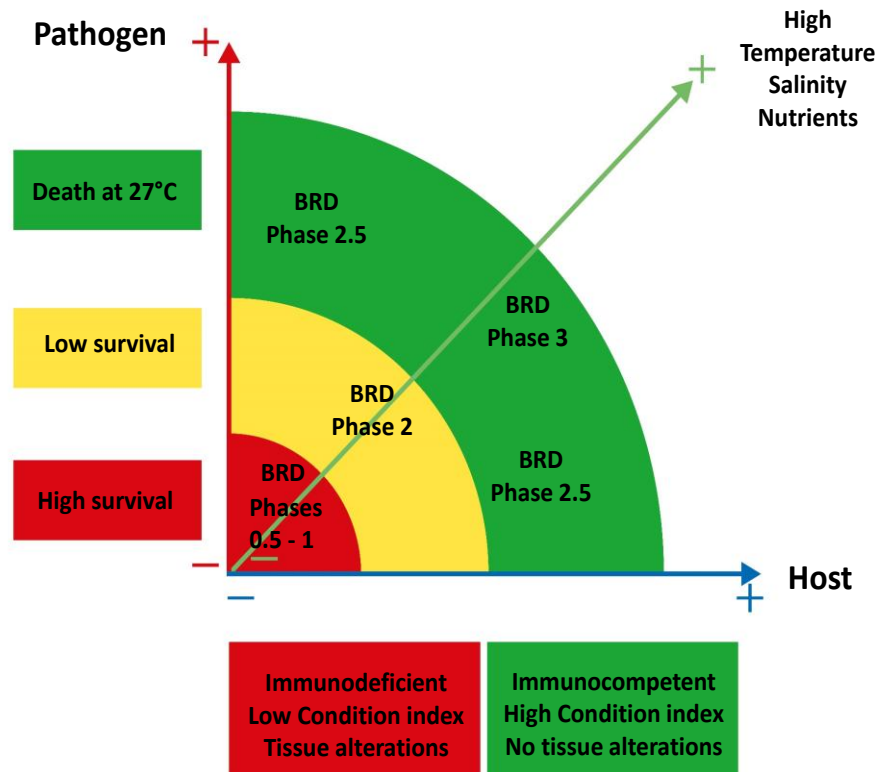


Figure 8. Synthesis of the Host-Pathogen-Environment system in the case of Brown Ring disease (BRD) in *R. philippinarum*.

Other environmental factors such as salinity can also modulate the *Vibrio*-clam interaction; in fact lower salinities enhance BRD development [24]. The quantity and quality of nutrients available also have a great impact on the condition index and on recovery processes of clam [8, 23]. Figure 8 integrates these different pressures on the host-pathogen

environment system. The theoretical individual-based model of Brown Ring Disease in Manila clams suggested also that the outcome of the host–parasite interaction is dependent on food supply and temperature [22, 25]. Simulations with increase temperature (1 or 2°C) favor generally disease development, indicating that climate warming might favor the spread of BRD, although this disease is a cold water vibriosis.

CONCLUSION

Brown Ring Disease in clams, caused by *Vibrio tapetis*, represents a unique model to explore how a bacterium, acting as a microparasite, can intimately alter bivalve physiology and ecology. Adopting an ecological approach to studying BRD will allow the coupling of various phenotypes (characteristic external signs, pathogen load and other life traits such as physiological and immunological patterns) to better understand host-parasite coevolution; moreover, BRD phenology could also be followed along with its response to climatic phenomena. New investigations into the role of microbiota on the host-pathogen-environment interplay represent an intriguing possibility for future research into ecological approaches to mollusc diseases.

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