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Understanding the role of Francisella halioticida in mussel mortalities in France: an integrative approach

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Abstract :

Since 2014, mass mortalities of mussels Mytilus spp. have occurred in production areas on the Atlantic coast of France. The aetiology of these outbreaks remained unknown until the bacterium Francisella halioticida was detected in some mussel mortality cases. This retrospective study was conducted to assess the association between F. halioticida and these mussel mortalities. Mussel batches (n = 45) from the Atlantic coast and English Channel were selected from archived individual samples (n = 863) collected either during or outside of mortality events between 2014 and 2017. All mussels were analysed by real-time PCR assays targeting F. halioticida; in addition, 185 were analysed using histological analysis and 178 by 16S rRNA metabarcoding. F. halioticida DNA was detected by real-time PCR and 16S rRNA metabarcoding in 282 and 34 mussels, respectively. Among these individuals, 82% (real-time PCR analysis) and 76% (16S rRNA metabarcoding analysis) were sampled during a mortality event. Histological analyses showed that moribund individuals had lesions mainly characterized by necrosis, haemocyte infiltration and granulomas. Risk factor analysis showed that mussel batches with more than 20% of PCR-positive individuals were more likely to have been sampled during a mortality event, and positive 16S rRNA metabarcoding batches increased the strength of the association with mortality by 11.6 times. The role of F. halioticida in mussel mortalities was determined by reviewing the available evidence. To this end, a causation criteria grid, tailored to marine diseases and molecular pathogen detection tools, allowed more evidence to be gathered on the causal role of this bacterium in mussel mortalities.

Keywords : Francisella halioticida, Mussel mortality, Causal inference, Molecular epidemiology, Histology, 16S rRNA metabarcoding, Real-time PCR

1. Introduction

In addition to their ecological and ecosystem importance, as ecosystem engineers for instance (Borthagaray & Carranza 2007, Buschbaum et al. 2008), farmed mussels are a significant food source for many aquatic and terrestrial animals including humans. For this reason, mussels have significant economic value in Europe and more generally in the world (FAO 2020). The French blue mussel industry represents thousands of jobs and France ranks 2nd in Europe in terms of the quantity of mussels produced per year (≈ 61,000 tonnes) but 1st in terms of the financial value of mussels (≈ €138,000,000) (FAO 2022, Agreste 2023). Since 2014, several mass mortality outbreaks in mussels (*Mytilus edulis, M. galloprovincialis* and their hybrids) have occurred in different production areas along the French coasts. These mortalities were highly heterogeneous in space and time (Béchemin et al. 2015; Allain & Bernard 2016, Charles et al. 2020a). These unusual mass mortalities, with rates up to 90-100%, have resulted in significant losses for many farmers with severe economic consequences for the whole French mussel farming industry.

In contrast to those mortality events in other bivalve species, such as Pacific oysters (*Crassostrea gigas*), very few mass mortalities of *Mytilus* spp. have occurred along French coasts in recent decades. Many historic mortalities in mussel farming along the European Atlantic coast have always been mainly attributed to predation, parasitism or unusual extreme temperature conditions (Robert & Le Moine 2003, Arzul et al. 2014, Mille 2017). Conversely, the aetiology of the mass mortality outbreaks that have been occurring since 2014 remains unidentified despite that probable risk factors were systematically reviewed (Lupo et al. 2021). Several studies, including a three-year project called MORBLEU, investigated the potentially aggravating factors associated or correlated with the mussel mortalities observed since 2014 (Pépin et al. 2016, Charles et al. 2020a). These factors included environmental influences, genetic background, ploidy levels of mussels and the possible pathogenesis link with infectious agents (Pépin et al., 2016). These studies have demonstrated that mussel mortalities were influenced by specific locations, with occurrences

mainly in the winter and spring during hot and rainy weather conditions. They have also found to coincide with phytoplankton blooms and changes in bacterial equilibrium (Polsenaere et al. 2017, Pépin et al. 2020). Genetic diversity of mussel did not differ from that of the previous years (Pépin et al. 2018, Simon et al. 2020). However, the species *Mytilus edulis* was more susceptible than *M. galloprovincialis* or hybrids although a possibility of a potential genetic adaptation was hypothesised (Dégremont et al. 2019). The potential role of disseminated neoplasia or genomic abnormalities in causing the disease was also investigated (Benabdelmouna & Ledu 2016, Benabdelmouna et al. 2018), as they could lead to death (Elston et al. 1988, Ciocan & Sunila 2005). The low prevalence observed during the different mortality events alone could not explain the high mortality rates obtained (Charles 2019, Charles et al. 2020a, Baez-Ortega & Murchison 2022). As no major pathogen protozoan like *Marteilia* sp., metazoan like trematods or lesions suggesting viral presence were detected, bacterial involvement was therefore the main probable etiological cause of the episodes of mass mortalities of French mussels. Several studies have questioned this hypothesis particularly the role of *Vibrio splendidus* species. However, their findings proved inconclusive (Béchemin et al. 2015, Charles et al. 2020b). Following this, Charles et al. (2020a) reported the existence of pathological conditions without any identifiable inducing agent and underlined the possibility of an unknown or uncultivable bacterial agent. Recently, the presence of the bacterium *Francisella halioticida* (Brevik et al. 2011) was detected by Polymerase Chain Reaction (PCR) and *In Situ* Hybridization (ISH) in mussels (*Mytilus* spp.) experiencing mortality in France (Charles et al. 2020c) and in archived samples of mussels in the United Kingdom (Cano et al. 2022), respectively.

The bacterium *F. halioticida* was originally identified as the causative agent of mass mortality in giant abalone (*Haliotis gigantea*) in Japan (Kamaishi et al. 2010, Brevik et al. 2011). Afterwards, infection with *F. halioticida* was associated with disease and mortality among Yesso scallops (*Mizuhopecten yessoensis*) in Canada (Meyer et al. 2017) and Japan (Kawahara et al. 2018). In

these studies, *F. halioticida* was observed in microscopic lesions and haemocyte infiltrations as well as in visible adductor muscle lesions. More recently, the pathogenicity and high virulence of *F. halioticida* in Yesso scallops have been more precisely demonstrated (Kawahara et al. 2019), showing that this bacterium has a pathogenic effect in both gastropod and bivalve mollusc species. It has most certainly been underestimated during unexplained past mortality events that may have affected shellfish across the world due to a lack of appropriate culture media for bacterial isolation (Kamaishi et al. 2010). It is impossible to culture *F. halioticida* with ordinary culture media such as marine Zobell agar or Marine agar which are routinely used to isolate bacteria from marine aquatic organisms, and this bacterium needs a specific medium and conditions to grow (Kamaishi et al. 2010, Kawahara et al. 2018). This aspect could explain the lack of detection in France, since marine Zobell agar or Marine agar media are routinely used by the national mollusc disease surveillance network, Repamo (Dufour & Hendrikx 2009).

Demonstrating a causal link between a pathogen and a disease is not always obvious and it is not only based on its ability to reproduce the clinical forms of disease. Many disease outcomes, notably in a marine context, are a result of the interplay and balance between multiple factors, similar to the situation with French mussel mortalities. Historically, Koch's postulates (Koch 1882) set the standard for proving the role of a microorganism in a disease. For the four postulates to be fulfilled in their current form, the identified microorganism must (1) be present in all cases of the disease; (2) be isolated from diseased patients; (3) cause disease when reintroduced to a healthy susceptible animal model; and (4) then be isolated again from the new host. However, these postulates present some limitations, especially if multiple factors combine to cause a disease or when pathogens may be difficult or impossible to grow in culture, which is often the case in marine systems (Ritchie et al. 2001). Different authors have proposed adapting these postulates to evaluate the evidence of causation in epidemiology (Hill 1965, Evans 1976), particularly by using

observational studies (Dohoo et al. 2010) or including new molecular tools for pathogen detection (Fredricks & Relman 1996, Fedak et al. 2015).

Hence, due to the recent detection of *F. halioticida* DNA in mussels (Charles et al. 2020c, Pépin 2020, Cano et al. 2022) and its confirmed pathogenicity in scallops (Kawahara et al. 2019), a retrospective study was conducted to assess the association between this causal candidate pathogen and the onset of mass mussel mortality in France since 2014, using a set of causation criteria adapted both to the context of multifactorial marine diseases and molecular tools for pathogen detection.

2. Materials & methods

2.1. Study design

This study used a retrospective case-control observational design, with the mussel (*Mytilus* sp.) batch as the epidemiological unit. A batch was defined as a group of mussels farmed in the same region in the same year, i.e. exposed to the same space and time conditions. Mussel batches were selected from the archived collection of samples of mortality cases submitted within the context of the national mollusc disease surveillance network Repamo (Dufour & Hendrickx 2009) and dedicated regional research programs on mussel mortalities (Travers et al. 2016, Charles 2019). Case batches were groups of mussels sampled during increased mortality events notified by farmers to the local competent authority (Council directive 2006/88/EC) and control batches were groups of mussels sampled outside of any mortality reports by farmers in different French production areas, i.e. all the “non-cases” recorded in the collection. Only the mussel batches sampled in three production regions located along the Atlantic coast and English Channel (i.e. Charente-Maritime, Southern Vendée and Northern Brittany) from 2014 to 2017 were included in this study (Fig. 1). The study was not targeting mussel batches sampled in other areas or outside the time window between 2014 and 2017 as the number of available samples from other areas or

years was too low. Data related to the location and year of production were collected at the batch level. Each batch was made up of several mussels, for which the individual health status was registered as either moribund or alive.

2.2. Laboratory analyses

All mussels included in the present study were analysed by real-time PCR and in addition, some were analysed by 16S rRNA gene metabarcoding sequencing and/or histology (Fig. 2).

2.2.1. Detection of *Francisella halioticida* DNA by real-time PCR

For each mussel, approximately 50 mg of each target tissue (a mixture of gills/mantle, posterior adductor muscle and digestive gland) was subjected to DNA extraction using a QIAamp DNA minikit® (Qiagen, Courtaboeuf, France) as per the manufacturer's protocol for blood or body fluids. The quality and quantification of the DNA were checked with the NanoPhotometer® N60 (Implen™ GmbH, Munich, Germany) and then the DNA was stored at -20°C. DNA extracts were diluted to a final concentration of 5 ng/µl before carrying out the real-time PCR assay. The presence of *F. halioticida* was investigated by using a TaqMan® real-time PCR assay (Charles et al. 2021) on DNA extracts from different mussel tissues. Amplification reactions were performed on a gradient thermal cycler CFX96 (Bio-Rad, Hercules, California, USA) using a 25 µl reaction volume containing 12.5 µl of ready-to-use SsoAdvanced™ Universal Probes Supermix 2X (Bio-Rad), 9.25 µl of purified water, 2 µl of DNA sample (replaced with 2 µl of purified water in the negative control), 0.25 µl of rpoB-Fw (20 µM), 0.5 µl of rpoB-Rev (20 µM) and 0.5 µl of probe (10 µM). Each assay included negative and positive control reactions; the *F. halioticida* DNA used for the positive control originated from colonies of *F. halioticida* isolated from diseased Yesso scallops (*Mizuhopecten yessoensis*) was kindly provided by Gary Meyer (Fisheries and Oceans Canada Nanaimo, BC, Canada); this positive control was diluted in order to use it close to the limit

of detection of the method specified by Charles et al. (2021). Thermal cycling conditions consisted of 95°C for 3 min followed by 40 cycles of 95°C for 15 s and 60°C for 30 s; fluorescence in the FAM channel was acquired during the annealing/extension stage.

2.2.2. Histology and *in situ* hybridization

The shell of each mussel was opened by cutting the adductor muscles and the soft tissues were removed. A 5 mm thick transverse section of mussel containing all the main tissues (mantle, digestive gland, gonads and gills) was excised, fixed for 24-48 h in Davidson's seawater fixative and then transferred to 70% ethanol before processing as per the standard paraffin inclusion protocol. Embedded paraffin blocks were then sectioned (2-3 µm) and stained with haematoxylin and eosin. Histological sections were examined for pathological conditions under light microscopy.

In addition, *in situ* hybridization (ISH) was performed on 19 individuals to confirm the presence of *F. halioticida* inside the mussel tissues. The protocol followed for ISH was adapted from Kamaishi et al. (2010) and Meyer et al. (2017). The three specific oligoprobes, Megai-110r, Megai-230r and Megai-870r, were purchased with 3' digoxigenin labelling. The 5 µm thick tissue sections were mounted on silane-prep™ slides (Sigma, France) and were then dewaxed, rehydrated and treated with proteinase K (100 µg ml^{-1} in TE buffer [Tris 50 mM, EDTA 10 mM]) at 37°C for 10 min. Slides were dehydrated by immersion in an absolute ethanol bath and air-dried. Sections were then incubated with 100 µl of hybridization buffer (50% formamide, 10% dextran sulfate, 4× Saline-sodium citrate buffer [0.06M Na_3 citrate, 0.6M NaCl, pH 7], 250 µg ml^{-1} yeast tRNA and 10% Denhardt's solution) containing 5 ng μl^{-1} of digoxigenin-labelled probes (Eurogentec). Target DNA and digoxigenin-labelled probes were denatured at 95°C for 5 min. and the hybridization was carried out overnight at 42°C. Sections were washed in 2×SSC at room temperature (RT) (2×5 min) and in 0.4×SSC at 42°C (10 min.). Tissues were then blocked for 10 min. at RT in the dark

with PBS 1X with hydrogen peroxide (3% in PBS) and washed in PBS 1X (2x5 min). Specifically, bound probes were detected using a peroxidase-conjugated mouse IgG anti-digoxigenin antibody diluted to 1 x 10^{-2} in PBS (1h, RT). Excess antibody was removed by two washes in PBS 1X. Slides were incubated in diaminobenzidine in the dark according to the manufacturer's instructions. Slides were counterstained for 30 s with haematoxylin, dehydrated with ethanol, mounted in Eukitt resin and then they were observed using a BX 50 microscope (Olympus). Negative controls included samples without a digoxigenin-labelled probe in the hybridization mixture or without antibodies during the revelation step. The positive control consisted of *Patinopecten yessoensis* infected with *F. halioticida* originating from Canada (kindly provided by G. Meyer).

2.2.3. Detection of *Francisella halioticida* DNA from 16S rRNA gene metabarcoding sequencing

DNA extraction

One hundred and seventy-eight (178) mussel samples from 27 batches were tested individually for the detection of *F. halioticida* DNA based on 16S rRNA gene Illumina® sequencing. Eighty-four individuals from 20 batches were subjected to DNA extraction from a mixture of gills and mantle according to the protocol described above for the detection of *F. halioticida* DNA by real-time PCR. Ninety-four individuals from seven other batches were subjected to DNA extraction from powder extract (50 mg) obtained with mussel in toto grinding after flash-freezing in liquid nitrogen. Subsequent DNA extraction steps were carried out as specified above in the real-time PCR section. DNA quality and quantification were carried out using a NanoDrop™ 2000 spectrophotometer (Thermofisher, Illkirch-Graffenstaden, France) and then stored at -80°C. DNA extracts were diluted to a final concentration of 10 ng μL^{-1} before PCR library preparation.

Library Preparation and Sequencing

A preliminary 16S Illumina metabarcoding study using part of the mussel samples from 2014 and 2015 revealed the presence of numerous reads affiliated with *F. halioticida* in the sequencing run (Pepin 2020). DNA libraries were constructed by two-step PCRs using 16S (V3-V4) metagenomic Illumina Sequencing Library preparation kits. Amplicons from the V3-V4 region of the 16S rRNA gene were produced using bacterial forward 343F (TACGGRAGGCAGCAG; Liu et al. 2007) and reverse 784R (TACCAGGGTATCTAATCCT; Andersson et al. 2008) primers and the P5 and P7 MiSeq™ adapters. The PCR conditions for the target gene were optimized with Phusion™ DNA Polymerase (Phusion® High-Fidelity DNA Polymerase, Thermo Fisher Scientific, VWR International - Fontenay-sous-Bois, France). Amplified samples were purified using the MinElute PCR Purification Kit (QIAgen) and were shipped to an external sequencing facility (Get-PlaGe, INRA, Toulouse). The constructed libraries were subjected to the sequencing of 250 paired-end bases using the Miseq™ Reagent Kit V3 on the Miseq™ system (Illumina).

Bioinformatic Analysis

The bioinformatic analysis was based on a preliminary study which revealed the presence of reads affiliated by BLASTn (Altschul et al. 1990) to the taxon *F. halioticida* (Pépin 2020) in the sequencing data, using the FROGS workflow (Find Rapidly OTU with Galaxy Solution, Escudié et al. 2018). These fragmentary data justified the implementation of the Amplicon Sequence Variant (ASV) generation method, ASV-based metabarcoding, using the R-based Divisive Amplicon Denoising Algorithm-2 (DADA2) pipeline (Callahan et al. 2016). The DADA2 workflow, an error-model based method for ASV inference was used to process the 16S rRNA gene amplicon data without resorting to the customary construction of molecular OTUs based on clusters of sequencing reads. This new approach has a finer resolution than the FROGS workflow (Callahan et al. 2017). A dedicated workflow was then designed to compare ASVs (426 bp) to the *F. halioticida* reference sequence published by Brevik et al. (2011) (GenBank accession number

JF.290376, *Francisella halioticida* strain Shimane-1 16S ribosomal RNA, partial sequence) and to detect and count reads affiliated with *F. halioticida* with a 100% coverage and similarity. A custom homemade pipeline was implemented (Quintric, pers. com.): Cutadapt to remove the primers, BBMap Merge to merge the R1/R2 pairs, Bowtie2 to create an index on the 16S sequence of *F. halioticida* and to map the reads of the samples merged on the *F. halioticida* index and Samtools view to extract the number of mapped sequences.

In the present study, the term "16S rRNA gene ASV-based metabarcoding" is replaced by "16S rRNA metabarcoding".

2.3. Statistical analyses

2.3.1. Variable construction and description

Individuals were considered positive for *F. halioticida* by PCR if they showed a fluorescent signal exceeding the fluorescent background level, regardless of the threshold cycle (Ct) obtained as recommended by the French norm U47-600-1 (2015). For the 16S rRNA metabarcoding, an individual was considered positive for *F. halioticida* DNA at a threshold of 100 reads. This arbitrary threshold was established as a value more than two times higher than the reads number with a 95% confidence interval (CI) obtained with extraction blank controls, in order to minimize the false positive counts (Ficetola et al. 2016) and to take into account the absence of a positive control in the 16S rRNA metabarcoding implementation (Alberdi et al. 2018).Quantitative variables were built using the proportion of positive individuals in which the DNA of *F. halioticida* had been detected by either PCR or 16S rRNA metabarcoding, or histological lesions had been observed. Qualitative variables were also built. A batch was considered positive for *F. halioticida* by PCR or 16S rRNA metabarcoding if at least one mussel was positive. A batch was defined as negative when all individuals were negative. A batch was considered to have histologically observed lesions if granuloma, infiltration or necrosis were observed in at least one mussel.

Variables were described in terms of frequency distributions (qualitative data) or the median and interquartile range (IQR) (quantitative data), classified by the outcome variable (case batches vs control batches).

2.3.2. Agreement estimation between methods

Fleiss' kappa was calculated to assess the agreement between the three laboratory methods in terms of detecting bacteria DNA or associated lesions in individuals (Fleiss et al. 2003) and the Kappa coefficient was calculated to assess the agreement between the two molecular laboratory methods, using Landis & Koch's (1977) interpretation.

2.3.3. Association between mortality and previously built variables

Statistical analyses were conducted at the batch level. The relationship between *F. halioticida* DNA detection and the risk of mortality was explored using individual *F. halioticida* DNA detection values, i.e. PCR Ct values of the digestive gland and the numbers of 16S rRNA metabarcoding reads. These variables were converted into categorical variables broken down into three or four categories; the cut-offs for the categories were chosen based on the respective variable distributions. Proportions of individuals in each categorical variable were compared between the case and control batches using a Chi-Square test with a statistical significance level $p = 0.05$. Thus, associations between mortality and *F. halioticida* detection by several laboratory methods were investigated at the batch level by comparing the frequency of *F. halioticida* detection in the cases with the frequency of *F. halioticida* detection in the controls. The strength of these associations was measured using the odds ratio (OR) with a 95% CI, using univariate logistic regression, with a statistical significance level set to $p = 0.05$. The potential associated variables investigated were region, year, season, proportion of PCR positive individuals per batch, proportion of the 16S rRNA metabarcoding positive individuals per batch and proportion of individuals with different types of histological lesions per batch. Due to very low numbers of batches observed in specific years, years

were grouped according to the perceived intensity of mortality for the given year (Normand et al. 2021).

A categorization of continuous quantitative variables was realised based on their quartile values to examine their relationship with the outcome variable. If the linearity assumption was not met, the appropriate shape was determined by merging logical categories or those that reflected changes in regression estimates. Associations were considered as statistically significant if the p-value of Wald's type 3 test was < 0.05. The minimum detectable OR for logistic regression with a single binary covariate was calculated with 80% statistical power and a 95% significance level, using the number of cases and controls in the study (Demidenko 2007).

2.3.4. Statistical tools

All statistical analyses were performed using the R Statistical Software (v4.3.0; R Core Team 2023). Logistic regression was carried out using the glm function of the stats package (R Core Team 2023), kappa coefficients were calculated using the raters package (Giardello et al. 2022) and the map was generated using the ggplot2 package (Wickham 2016).

2.4. Establishment of a grid of causation criteria adapted to the marine context and modern pathogen detection tools

Due to the difficulty to apply Koch's postulate, especially when the potential pathogen agent was impossible to grow or difficult to cultivate, a grid of six causality criteria was proposed, based on the combination of two existing sets of criteria:

1- the first one was based on the use of molecular tools and could be considered as a molecular form of Koch's postulate. It was established by Fredricks & Relman (1996) and based on the sequencing of microbial nucleic acids;

2- the second one relied on a combination of basic epidemiological observations and biological characterization established by Dohoo et al. (2010) and adapted from the criteria given by Hill (1965) and Evans (1976).

These six criteria are listed in Table 7. For each criterion, evidence from previous studies in the literature and the present study was supplemented (Table 7).

3. Results

3.1. Sample description

A total of 45 sampled batches comprised of 863 mussels were included in this study, of which 26 batches containing 446 individuals were cases, and 19 batches made up of 417 individuals were controls. Their characteristics are summarized in Table 1. The sampled batches were made up of a range from 10 to 40 mussels, with a median of 20 individuals sampled per batch. The median proportion of moribund mussels per batch was 0.31 (IQR = 0.89) in the case batches, and 0 (IQR = 0) in the control batches.

3.2. Laboratory analyses

Individual level

All 863 individuals were analysed by real-time PCR targeting *Francisella halioticida*. Among these, 185 were analysed by histology, and 178 by 16S rRNA metabarcoding as described in Fig. 2. A total of 75 individuals were analysed by the three laboratory methods.

Both real-time PCR or 16S rRNA metabarcoding detected *F. halioticida* DNA in both regions studied as well as in different years and as early as 2014 (Table 1). However, bacterium DNA was not detected in all batches sampled in a mortality context (Table 2). The presence of *F. halioticida* DNA was detected in 281 and 34 individual mussels by real-time PCR and 16S rRNA

metabarcoding, respectively, of which 83% (233/281) by real-time PCR and 79% (27/34) by 16S rRNA metabarcoding had been sampled during a mortality event (Table 2). Whether by real-time PCR or 16S rRNA metabarcoding, case batches showed higher proportions of individuals in higher levels of *F. halioticida* DNA copy numbers than control batches (Chi square test = 105.6, $p < 0.0001$ and Chi square test = 9.37, $p < 0.01$, respectively; Table 3). Specifically, 94% of the individuals in the control batches showed an absence of detection of bacterium DNA (no Ct values).

Histological analyses revealed the presence of certain parasites such as *Marteilia refringens* type M (molecular data not shown) or some sporocyst trematods but their prevalence was very low (Supplementary material Table S1). Some lesions were observed, especially on individuals collected within a context of mortality (Table 2 and Supplementary material Table S1) and detected as positive for *F. halioticida* by real-time PCR. These lesions were mainly necrosis and haemocyte infiltration of connective mantle and digestive gland tissues (Fig. 3a). In some cases, bacteria proliferation was observed in the connective tissues of the mantle and digestive gland (Fig. 3b). Granulomas were noted but only on 16 individuals (Fig. 3c), of which 11 were also positive for *F. halioticida* by real-time PCR and sampled during a mortality event. ISH was used to confirm the presence of *F. halioticida*. Eight individuals that tested negative in real-time PCR were also negative in ISH. Six individuals that were strongly positive in real-time PCR ($Ct < 30$) showed specific labelling of the bacteria inside host cells in the connective tissue of the gonads, digestive gland and mantle (Fig. 3d and 3e) and six individuals did not show any specific labelling whereas they were weakly positive in real-time PCR ($Ct > 33$).

Agreement between the three laboratory methods was fair (Fleiss kappa coefficient = 0.24, 95% CI [0.11-0.37], $p = 0.001$), calculated on 75 individual mussels (Table 4). A fair agreement was also observed between the PCR and 16S rRNA metabarcoding results (kappa coefficient = 0.24, 95% CI [0.09-0.38], $p < 0.001$), calculated on 178 individuals (Table 5).

Batch level

The median proportion of PCR positive individuals per batch was 0.59 (IQR = 0.70) in the cases and 0.07 in the control batches (IQR = 0.14) (Fig.4A); this proportion was 0.33 in the cases (IQR = 0.40) and 0 (IQR = 0.03) in the control batches for 16S rRNA metabarcoding (Fig. 4B). The median proportion of individuals with a histologic lesion per batch was 0.68 (IQR = 0.50) in the cases and 0.13 (IQR = 0.25) in the control batches (Fig. 4C).

3.3. Risk factor analysis

The results are summarized in Table 6. The risk of mortality was 11.6 times higher when positive 16S rRNA metabarcoding results were observed in the mussel batch ($p = 0.009$). In particular, the risk of mortality was increased by 19.5 times if a proportion of 16S rRNA metabarcoding positive individuals greater than 10% was observed in the batch ($p = 0.003$). Mussel batches with a proportion of PCR positive individuals higher than 20% also had 7.08 times more risk of mortality ($p = 0.005$). Batches with observed necrosis in histological examination were 25 times more likely to have been sampled during a mortality event ($p = 0.01$).

The region, year and observation of undefined histological lesions were not statistically associated with the probability of the mussel batch being sampled during a mortality event (Table 6).

The sample size of this study enabled the detection of a minimum OR of 2.33 with 80% statistical power, meaning that only strong associations between mussel mortality and the various variables explored could be identified.

3.4. Potential evidence provided for mussel mortality causation

Table 7 summarizes the results obtained in the present study, classified according to six causality criteria (numbered from 1 to 6). The present study was enabled to fulfil three criteria (no. 1, 3 and 5). Three criteria could not be evaluated (criterion 6) or were only partially evaluated (criteria 2

and 4), mostly due to the study design. As it was a retrospective study based on molecular detection of the bacteria in mussels sampled in the field, the time sequence (part of criteria 2) and experimental evidence (criterion 6) could not be demonstrated. In addition, as *F. halioticida* is an emerging pathogen in mussels, it is difficult to know its real geographical distribution. However, with the integration of literature data, criterion 4 was almost fulfilled.

4. Discussion

Although the mussel mortalities that have occurred since 2014 in France are a real problem for mussel farmers, no clear aetiology has yet to be identified to explain them (Polsenaere et al. 2017) although, some factors that favour their occurrence such as warm and rainy winters have been identified (Pépin et al. 2019). These mortalities appear to have multifactorial origin with various risk factors. One of them could be a pathogenic agent. Our study found no major pathogen in histology except bacteria infection. Bacteriological analyses carried out on these individuals using the classic bacteriological medium (Marine Agar) mainly revealed the presence of *Vibrio splendidus* strains (data not shown) although its presence was not correlated with field mortality observation (Pépin et al. 2019, Charles et al. 2020b). Therefore, it is hypothesized that *Francisella halioticida*, a bacterium whose DNA was recently detected in France, could potentially be one of the causes of these mortalities (Charles et al. 2020c).

To better understand its real role, the present retrospective study was implemented to assess the association between mussel mortality events occurring in France since 2014 and the presence of these bacteria, partly inferred via detection of its DNA. A multiple-testing methods approach was used combining both molecular and histological technics to confirm a real infection of mussel tissues by visualising the bacterium rather than solely DNA detection (Burreson 2008, Aranguren and Figueras 2016). Using various diagnostic methods may reveal some differences in the analyses results especially for samples with low infection rates as it was observed in our study given that

the methods present difference in analytical procedures and different diagnostic specificities and sensitivities; additionally, some of these technics such as histology or real time PCR are routinely employed while others like 16S rRNA metabarcoding are more challenging to implement. However, although there were some discrepancies according to the techniques used, the results obtained demonstrated that the risk of a mortality event was higher when *F. halioticida* DNA was detected in proportions of mussels in the sampled batch, whether by PCR or 16S rRNA metabarcoding. Moreover, its detection was confirmed by ISH for strongly positive individuals in real-time PCR (Ct < 30) and 16S rRNA metabarcoding, which showed specific labelling of the bacteria inside host cells in connective gonad, digestive gland and mantle tissues.

Although bacterial identification and detection mainly relied on sequence-based methods in this study because of the absence of a cultivated bacterium, these findings suggest a potential causal relationship between *F. halioticida* infection and mussel mortalities. Thus, a combination of different criteria for causation (Table 7) adapted both for observational studies (Dohoo et al. 2010) and the use of molecular tools for pathogen detection (Fredericks and Relman 1996, Fedak et al. 2015) were applied to assess the relationship between the bacterium *F. halioticida* and mussel mortalities.

Criterion no. 1: Observations in diseased mussels

Our retrospective study and the literature showed that *F. halioticida* DNA was mainly detected during mussel mortality events whether by PCR or 16S rRNA metabarcoding (Charles et al. 2020c, Cano et al. 2022). ISH showed that bacteria DNA was inside PCR positive tissues and that these tissues (digestive gland, gills and mantle) presented necrosis lesions and sometimes granulomatous inflammation. Moreover, our study estimated the strength of the association between *F. halioticida* detection using several laboratory methods and mussel mortality, by calculating the OR measurement. Significant odds were observed for mussel batches showing positive 16S rRNA

metabarcoding results (OR = 11.6, p = 0.009), a proportion of 16S rRNA metabarcoding positive individuals greater than 10% (OR = 19.5, p = 0.003), a proportion of PCR positive individuals greater than 20% (OR = 7.08, p = 0.005), or histologically observed necrosis (OR = 25.0, p = 0.01). Thus, our results demonstrated a strong association between the detection of *F. halioticida* DNA and the observation of mussel mortality, which supports the hypothesis that this bacterium is involved in mussel mortality events.

Criterion no. 2: Observations in healthy mussels and disease progression

Batches collected during mortality events showed higher proportions of individuals in higher levels of *F. halioticida* DNA copy numbers than in the control batches, whether by real-time PCR or 16S rRNA metabarcoding. Conversely, 94% of individuals in the control batches showed an absence of detection of *F. halioticida* DNA, i.e. no Ct values. In addition, no ISH labelling and few lesions were observed in tissues from the control mussels. A similar relationship was also observed with the same bacterium species in Yesso scallops and with other virulent *Francisella* species in different fish species such as *Francisella noatunensis* subsp. *orientalis* infecting tilapia (Colquhoun & Duodu 2011, Kawahara et al. 2019). These data reinforce the causal link between the mussel mortalities described and the occurrence of *F. halioticida.*

The biological gradient could not be evaluated due to the study design which did not consider variations in the degree of batch mortality (i.e. mortality percentage) that might have made it possible to observe a gradient in terms of bacteria detection. However, accurate mortality estimations in mussels in farming conditions are not standardized (Lupo et al. 2021), which prevents any valid use of reported mortality rates to estimate a meaningful dose-response relationship in such a context.

Likewise, the time sequence could not be evaluated due to the choice of the retrospective study design in which it was not possible to assess if the exposure to *F. halioticida* preceded the mussel

mortality events. Meanwhile, previous studies (Travers et al. 2016, Pépin 2020) were used to document the temporality criterion. A same batch was sampled in different periods before and during mortality events and analysed by 16S rRNA metabarcoding to detect *F. halioticida* DNA. The results obtained showed that very few 16S sequence reads of *F. halioticida* were detected before mortality, whereas 50% of the sampled mussels presented large numbers of reads during a mortality event. These data suggest a certain temporality in the exposure of mussels to *F. halioticida* but further studies will be necessary to provide additional support for this criterion.

Criterion no. 3: Plausibility

The family Francisellaceae comprises pathogens including the well-known human pathogen *Francisella tularensis* and members pathogenic to marine species like *F. noatunensis* or *F. orientalis,* associated with tilapia or Atlantic cod mortalities (Colquhoun & Duodu 2011, Ellingsen et al. 2011). The species *F. halioticida* was first described during marine mollusc (abalones and Yesso scallops) mortalities and was clearly identified as being responsible for these mortalities before it was detected in mussels (Kamaishi et al. 2010, Brevik et al. 2011, Meyer et al. 2017, Kawahara et al. 2019). Therefore, its detection during mollusc mortality events is not an isolated case; these bacteria were detected in different regions worldwide and is has been acknowledged that they have a negative effect on mollusc farming. In fish, *Francisella* DNA was detected in a specific organ associated with tissue lesions (Colquhoun & Duodu 2011). *Francisella halioticida* presents the same traits given that its DNA was mainly detected in different mussel organs presenting lesions. In addition, specific *Francisella* ISH confirmed the presence of the bacteria inside tissue lesions and sometimes in granuloma and not in the mussel healthy tissues included in the present study, which is consistent with previous studies in mussels (Charles et al. 2020c, Cano et al. 2022). These findings suggest a certain relationship between lesions and the presence of bacteria.

In the present study, *F. halioticida* did not induce specific macroscopic symptoms in mussels contrary to the pustules and ulcers observed on the muscle of Yesso scallops or on the foot of giant abalones (Brevik et al. 2011, Meyer et al. 2017). Meanwhile, even if no specific lesions were really observed in histology, tissular changes were noticed in organs determined to be infected by *F. halioticida* by PCR, such as the digestive gland or mantle. Haemocyte infiltration and necrosis were noted and, in some cases, granulomatous lesions were observed as previously described with francisellosis in other marine species (Brevik et al. 2011, Colquhoun & Duodu 2011, Meyer et al. 2017). Granulomatous lesions are often observed in animals infected by *Francisella* bacteria even if these lesions were not specific to this genus. Similar lesions can be present in other bacterial infections such as *Mycobacterium* or *Nocardia* infections (Colquhoun & Duodu 2011), and they are frequently observed in mussels, particularly in polluted areas (Sheir et al. 2013, Arienzo et al. 2019). However, this does not exclude the possibility that granuloma lesions could be induced by *F. halioticida*. In the present study, no real association was found between the presence of *F. halioticida* DNA and the observation of granuloma; this could possibly be due to the low number of individuals from control batches observed in histology (N = 20). As pointed out by Capelle et al. (2021), it would be interesting to carry out further specific studies on this point.

Another particularity of this genus is the existence of several subspecies within the same species which could exhibit high host specificity, such as *F. tularensis* (Hennebique et al. 2019). This possibility was suspected in *F. halioticida* by Kawahara et al. (2021) who suggested the possible existence of different genotypes of *F. halioticida* affecting either abalones or scallops. The same assumption has been made by Bouras et al. (2023) concerning *F. halioticida* strains affecting mussels. Several strains were isolated from mussels and they presented different biochemical and genetic differences suggesting the existence of genotypes/subspecies. Its aspect is currently under investigation and if the existence of genotypes/subspecies inside the species *F. halioticida* is confirmed, this would agree with the specific features of the genus *Francisella*.

Criterion no. 4: Reproducible evidence/consistency

Francisella halioticida has only recently been detected in mussels, and therefore few studies have been performed to understand its implication in mussel mortalities in France or in Europe but the recent development of a real-time PCR tool will help to develop such studies (Charles et al. 2021). To date, these bacteria were detected in France, as well as in the United Kingdom and the Netherlands (Cano et al. 2022, Marc Engelsma pers. comm.), in association with mussel mortality events.

Criterion no. 5: Study design and statistical issues

The case-control design of this observational study was suitable for identifying and measuring statistical associations between mussel mortalities and *F. halioticida* DNA detection. The control mussel batches selected were groups of mussels sampled in the same French production areas and years as the case batches, so that they would be representative of the exposure experience in the population that gave rise to the cases. We believe that this control selection is appropriate to limit selection and confounding bias. In this study, the minimum detectable OR was equal to 2.33. This calculation estimates the smallest association that, if it even exists, could be statistically detected in the present study. The estimated statistically significant OR were greater than 2.33, i.e. mussel batches showing positive 16S rRNA metabarcoding results (OR = 11.6), with a proportion of 16S rRNA metabarcoding positive individuals greater than 10% (OR = 19.5), or a proportion of PCR positive individuals greater than 20% (OR = 7.08). Thus, we believe the statistical power of our study was sufficient to detect if there is an effect of the presence of the bacteria DNA in mussels and whether the sample size of the study was adapted to our objective.

Criterion no. 6: Experimental evidence

In this study, we showed that the presence of *F. halioticida* DNA was associated with a higher probability of mussel mortality but it remains to be shown that this bacterium is pathogenic to this bivalve. It has been proven that *Francisella* spp. are pathogenic to marine organisms (Kamaishi et al. 2005, Soto et al. 2010): *F. halioticida* is pathogenic to giant abalone, *Haliotis gigantea* (Kamaishi et al. 2010) and Yesso scallops, *Mizuhopecten yessoensis* (Kawahara et al. 2019). Concerning mussels, with the recent isolation of *F. halioticida* strains on mussels, some experimental infections have begun and need to be continued in order to determine whether this bacterium can be classified as pathogenic to mussels (Bouras et al. 2023). If experimental pathogenicity of some strains is proven, the involvement of this bacterium in mortality events should be further investigated.

5. Conclusion

This study has provided the first information that supports the hypothesis of *Francisella halioticida* being a putative pathogen in mussel mortalities, something that has been regularly observed in France since 2014. The application of two sets of guidelines for causation provided an adapted framework to discuss causation inferred from both epidemiological information and molecular detection. Based on our study and literature data, three out of six of our criteria were clearly fulfilled: they mostly correspond to observations in diseased mussels (criterion no. 1), plausibility (criterion no. 3) and the study design as well as methodological issues related to Dohoo et al.'s (2010) criteria (criterion no. 5). However, further studies are needed to complete the present causation study, in particular to gather more information about these bacteria and to fulfil all the causality criteria. Criterion no. 6, corresponding to experimental evidence, could not be demonstrated due to our study design (detection of *F. halioticida* with molecular tools only) but with the recent isolation of the bacteria from French mussels (Bouras et al. 2023), different experiments could be performed to demonstrate the potential impact of this bacterium on mussels.

Moreover, with the development of a PCR tool targeting these bacteria (Charles et al. 2021), it will be easier to implement studies to determine the time sequence of the infection (criterion no. 2) as well as to detect the bacteria if mussel mortalities are observed in different countries (criterion no. 4).

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Table 1. Summarized numbers of cases and controls of the mussel samples at the individual (n=863) and batch levels (n=45) included in the study between 2014 and 2017, France. The positive or negative at the individual level is determined respectively by PCR, 16S rRNA metabarcoding, and histology. A batch is defined as positive if one individual or more from the batch is positive by each corresponding laboratory tests.

Variables and categories	**Context of the mortality event** No batches (individuals)		**PCR** No batches (individuals)		**16S rRNA metabarcoding** No batches (individuals)		**Histologically observed lesions** No batches (individuals)	
	Cases	Controls	Positive	Negative	Positive	Negative	Yes	No
Year								
2014	7 (130)	1 (10)	3 (20)	5 (120)	2 (3)	3 (35)	6 (61)	0 (29)
2015	5 (66)	4 (70)	9 (63)	0 (73)	4 (13)	2 (69)	1 (9)	0 (6)
2016	5 (50)	8 (217)	10 (40)	3 (227)	1 (2)	0 (8)	2 (20)	0 (0)
2017	9 (200)	6 (120)	15 (158)	0 (162)	9 (16)	6 (32)	10 (26)	5 (34)
Season								
Spring	19 (319)	15 (357)	28 (192)	6 (490)	13 (29)	7 (114)	12 (82)	5 (48)
Summer	1 (20)	0 (0)	1 (5)	0 (9)	0 (0)	0 (0)	0 (0)	0 (0)
Autumn	2 (40)	0 (0)	2 (32)	0 (8)	2 (4)	0 (2)	2 (10)	0 (3)
Winter	4 (67)	4 (60)	6 (52)	2 (75)	1 (1)	4 (28)	5 (24)	0 (18)
Region								
English Channel	12 (240)	6 (120)	16 (161)	2 (199)	9 (16)	6 (32)	11 (36)	5 (39)
Atlantic coast	14 (206)	13 (297)	21 (120)	6 (383)	7 (18)	5 (112)	8 (80)	0 (30)
Total	26 (446)	19 (417)	37 (281)	8 (582)	16 (34)	11 (144)	19 (116)	5 (69)

Table 2. Results of the individual laboratory tests (PCR, 16S rRNA metabarcoding and histology observations) according to the sampling context (case or control batches).

	Individuals from case batches	**Individuals from control batches**
PCR results		
Negative	213	369
Positive	233	48
16S rRNA metabarcoding **results**		
Negative	59	85
Positive	27	7
Histology results		
No observed lesions	52	17
Lesions	113	3
Granuloma observed		
No	151	18
Yes	14	2
Infiltration observed		
No	136	20
Yes	29	0
Necrosis observed		
No	72	19
Yes	93	1

Table 3. Distribution of individual levels of *F. halioticida* DNA detection values, i.e. individual real-time PCR Ct values of the digestive gland (n = 633) and number of 16S DNA reads numbers (n = 178) in case and control batches

F. halioticida DNA detection	Number of individuals from case batches (proportion)	Number of individuals from control batches (proportion)
real-time **PCR Ct value**		
No Ct	218 (0.54)	217 (0.94)
36.0 < Ct < 40	35 (0.09)	5 (0.02)
33.3 < Ct ≤ 36.0	57 (0.14)	5 (0.02)
Ct ≤ 33.3	91 (0.23)	5 (0.02)
Number of 16S reads		
< 10	43 (0.50)	62 (0.67)
10≤ reads number < 40	13 (0.15)	16 (0.14)
≥ 40	30 (0.35)	14 (0.15)

Table 4. Contingency table of test results of the selected individual mussels (n = 75) using real-time PCR, 16S rRNA metabarcoding and histology.

	16S rRNA metabarcoding positive	**16S rRNA metabarcoding negative**
PCR negative (n = 49)		
Histologically observed lesions	1	23
No lesion	3	22
PCR positive (n = 26)		
Histologically observed lesions	13	3
No lesion	2	8

Table 5. Contingency table of test results of the 178 individual mussels analysed by real-time PCR and 16S rRNA metabarcoding.

	16S rRNA metabarcoding	
PCR	positive	negative
positive	19	36
negative	15	108

Table 6. Potential associated variables with a mussel mortality event at the batch level, univariate logistic regression, Atlantic coast and English Channel, France, 2014-2017. OR stands for "Odds Ratio", 95% CI for "95% confidence interval", Ref for "reference group".

Variables and categories	**Case batches**	**Control batches**	**OR**	**95% CI**	**p**
Region					
English Channel	12	6	Ref		
Atlantic coast	14	13	0.54	0.15-1.82	0.327
Years					
2014; 2016	12	9	Ref		
2015; 2017	14	10	1.05	0.32-3.47	0.936
Season					
Other seasons	7	4	Ref		
Spring	19	15	0.72	0.16-2.87	0.652
PCR results					
Negative	5	3	Ref		
Positive	21	16	0.79	0.14-3.70	0.766
Histology results					
No observed lesions	2	3	Ref		
Lesions	16	3	8.00	0.96-86.72	0.061
16S rRNA metabarcoding results					
Negative	3	8	Ref		
Positive	13	3	11.60	2.09-86.52	0.009
Proportion of PCR positive individuals per batch					
≤ 20%	9	15	Ref		
> 20%	17	4	7.08	1.94-31.11	0.005
Proportion of 16S rRNA metabarcoding positive individuals per batch					
≤ 10%	3	9	Ref		
>10%	13	2	19.50	3.19-186.73	0.003
Proportion of individuals with histologically observed lesions per batch					
≤ 20%	2	3	Ref		
>20%	16	3	8.00	0.96-86.72	0.061
Histologically observed granuloma					
No	11	4	Ref		
Yes	7	2	1.27	0.19-11.0	0.808
Histologically observed necrosis					
No	3	5	Ref		
Yes	15	1	25.00	2.81-587.88	0.011
Histologically observed infiltration					
No	5	6	Ref		
Yes	13	0	31.90[1]	NA	NA

[1] This OR was calculated using the Haldane-Anscombe correction, but no CI 95% or p-value could be calculated and are noted NA (= not applicable).

Table 7. Authors' interpretation of the criteria grid for providing evidence of causation concerning the relationship between *Francisella halioticida* and mussel mortality (NA stands for "No evidence available")

Proposed Criteria	**Molecular tools criteria** (Fredericks and Relman, 1996)	**Observational epidemiology criteria** (Dohoo 2010)	**Evidence in the present study**	**Evidence in literature**
1. Observations in diseased individuals	(i) A nucleic acid sequence belonging to a putative pathogen should be present in most cases of an infectious disease. Microbial nucleic acids should be found preferentially in those organs or gross anatomic sites known to be diseased (i.e. with anatomic, histologic, chemical, or clinical evidence of pathology) and not in those organs that lack pathology.	Specificity of association	-Necrosis lesions and sometimes granulomatous inflammation in detected infected mussels by PCR (tissues: digestive gland, gills and mantle) -*Francisella halioticida* DNA presence in organs with lesions	*F. halioticida* DNA was detected in mussels with tissue lesions (Charles et al. 2020c, Cano et al. 2022)
			-Increased mortality probability for mussel batches showing positive results to 16S rRNA metabarcoding	*F. halioticida* is associated with mussels and other molluscs mortalities (Brevik et al. 2011, Meyer et al. 2017, Kawahara et al. 2019, Charles et al. 2020c)
		Strength of association	-Increased mortality probability for mussel batches showing positive results to 16S rRNA metabarcoding (OR = 11.6, CI 95% [2.09-86.5]) -Increased mortality probability for mussel batches showing a proportion of 16S rRNA metabarcoding positive individuals higher than 10% (OR = 19.5, C 95% [3.19-186.7]) - Increased mortality probability for mussel batches showing a proportion of PCR positive individuals higher than 20% (OR = 7.08, CI 95% [1.94-31.1])	NA

	(vi) Tissue-sequence correlates should be sought at the cellular level: efforts should be made to demonstrate specific in situ hybridization of microbial sequence to areas of tissue pathology and to visible microorganisms or to areas where microorganisms are presumed to be located.	/	-*F. halioticida* detection confirmed by ISH for individuals strongly positive in real-time PCR (Ct < 30), which showed specific labelling of the bacteria inside host cells in connective tissue of gonads, digestive glands and the mantle, and also by 16S rRNA metabarcoding for individuals with more than 1500 reads -*F. halioticida* ISH positive labelling in damaged tissues of diseased mussels	*F. halioticida* appears to be associated with the host's response and formation of granulomas (Charles et al. 2020c, Cano et al. 2022)
2. Observations in healthy individuals and disease progression	*(ii) Fewer, or no, copy numbers of pathogen-associated nucleic acid sequences should occur in hosts or tissues without disease.*	/	-No lesion in alive (i.e. assumed to be healthy) mussels. -No ISH labelling in tissue of control mussels.	Molluscs including mussels with no significative lesions presented negative labelling with *F. halioticida* probe in ISH (Meyer et al. 2017, Charles et al. 2020c)
			-Mussels from case batches showed higher proportions of individuals with higher levels of *F. halioticida* DNA copies. -94% of the individuals of the control batches showed no Ct values (i.e. absence of detection of *F. halioticida* DNA).	Observation of mollusc mortalities challenged with *F. halioticida* via bath exposure; no mortality in controls (Kawahara et al. 2019)
	(iii) With resolution of disease (for example, with clinically effective treatment), the copy number of pathogen-associated nucleic acid sequences should decrease or become undetectable. With clinical relapse, the opposite should occur.	/	NA	NA
	(iv) When sequence detection predates disease, or sequence copy	Dose-response relationship	NA	NA

	number correlates with severity of disease or pathology, the sequence-disease association is more likely to be a causal relationship.	Time sequence	NA	*F. halioticida* was detected before and during mussels mortality events (Travers et al. 2016, Pépin 2020)
3. Plausibility	(v) The nature of the microorganism inferred from the available sequence should be consistent with the known biological characteristics of that group of organisms. When phenotypes (e.g. pathology, microbial morphology, and clinical features) are predicted by sequence-based phylogenetic relationships, the meaningfulness of the sequence is enhanced.	Plausibility or coherence	-*F. halioticida* DNA detection in digestive gland or mantle was associated with tissue lesions in these organs (haemocyte infiltration, necrosis, granulomatous lesions) -*F. halioticida* ISH positive labelling in damaged tissues of diseased mussels	-Other bacteria from *Francisella* spp. associated with fish mortality (Colquhoun & Duodu 2011, Ellingsen et al. 2011) -*F. halioticida* involved in abalone and Yesso scallop mortalities (Brevik et al. 2011, Kamaishi et al. 2010, Kawahara et al. 2019, Meyer et al. 2017) -*Francisella* bacteria affect muscles and induce granuloma lesions in fish (Colquhoun & Duodu 2011, Junior et al. 2020) -possible existence of different strains and genotypes of *F. halioticida* affecting either abalones, scallops and also mussels (Kawahara et al. 2021, Bouras et al. 2023)
4. Reproducible evidence / consistency	(vii) These sequence-based forms of evidence for microbial causation should be reproducible.	Consistency	Detection in different French mussel production areas in several years	-Detection in France, the UK and in the Netherlands (Charles et al. 2020c, Cano et al. 2022, Marc Engelsma, pers. comm.)

5. Study design and statistical issues	Study design and statistical issues	-Case-control design, with selection of control mussel batches sampled in the same French production areas and years as the case batches	NA
6. Experimental evidence	Experimental evidence	NA	Experimental trials with one *F. halioticida* strain isolated from mussels did not lead to significant mortalities. Other trials will be performed with other strains (Bouras et al. 2023)

3

4

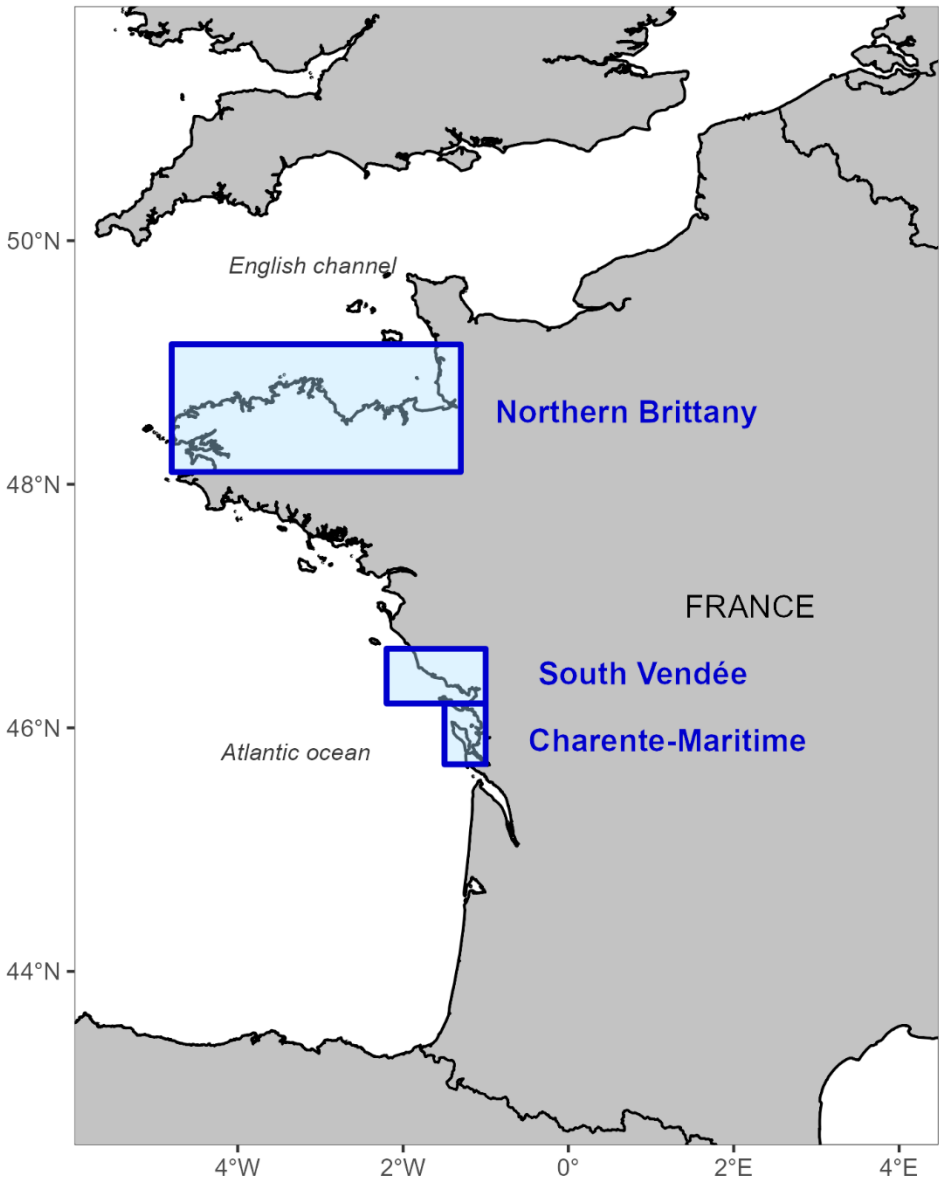

Fig. 1. Location of the studied population of mussels in Northern Brittany, South Vendée, and Charente-Maritime in France

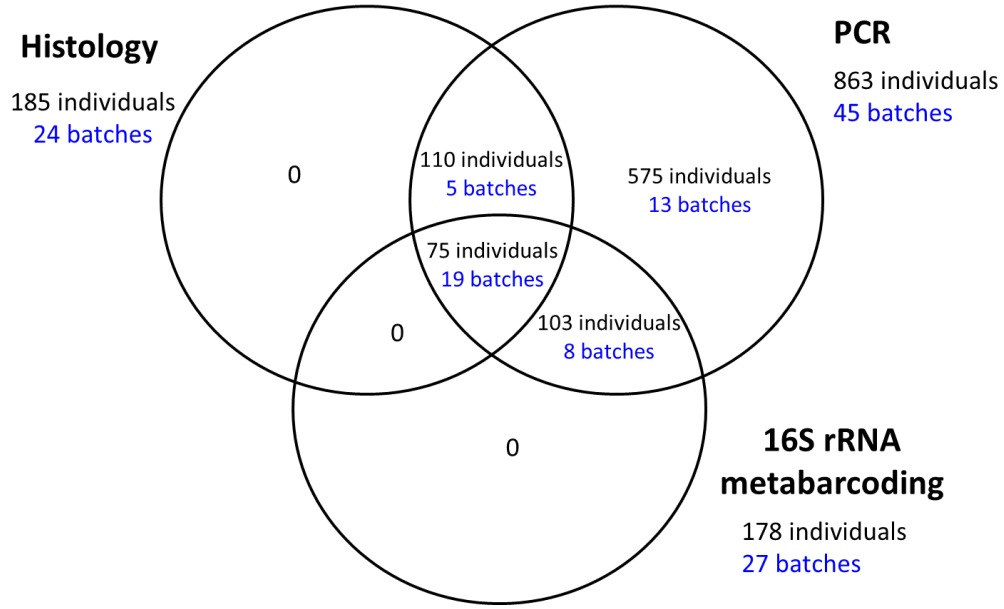

Fig. 2. Venn diagram with the sample number of mussel batches (n=45) and individuals (n = 863) respectively analysed by the three laboratory methods: real-time PCR, 16S rRNA metabarcoding and histology.

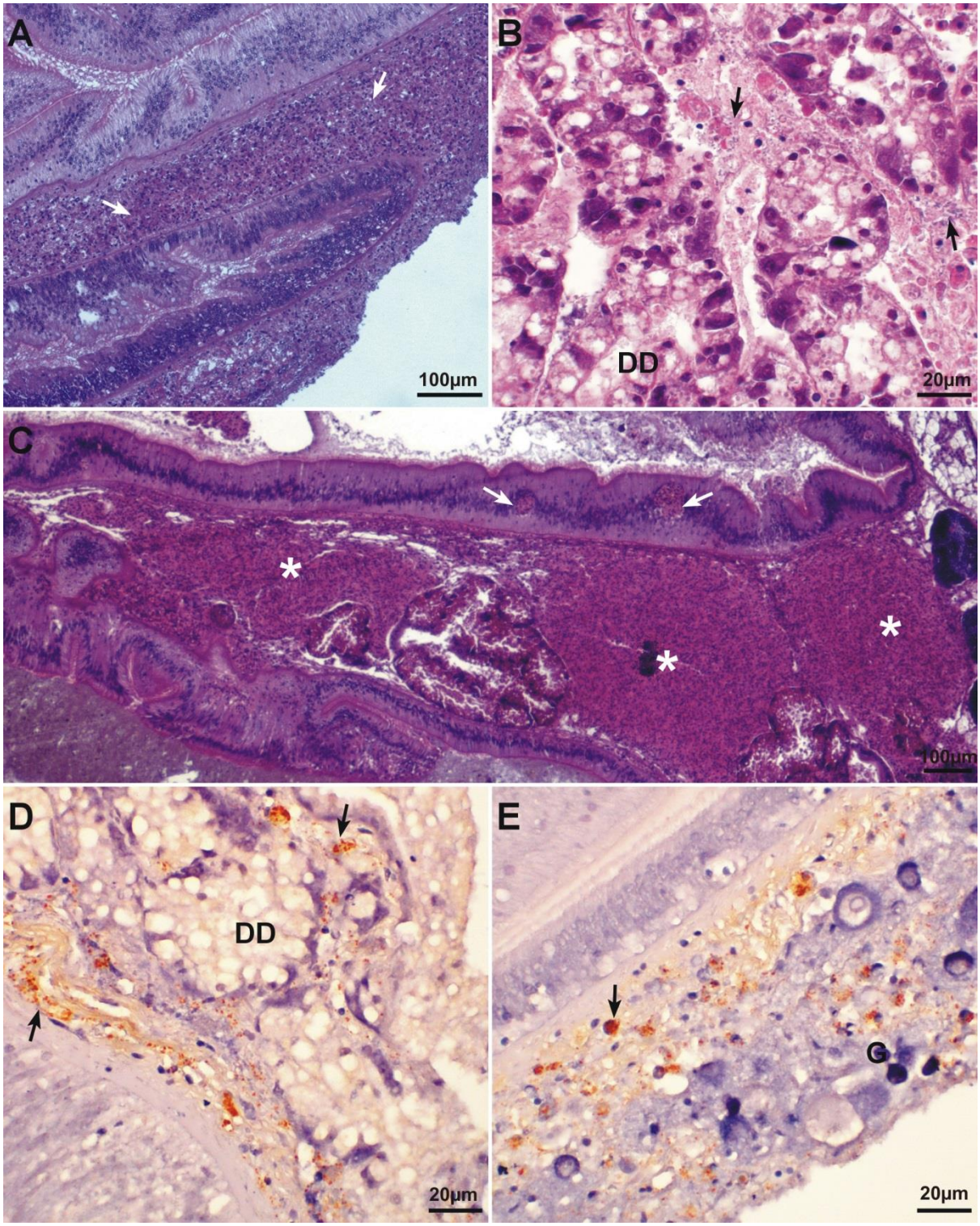

Fig. 3. Haematoxylin and eosin-stained tissue sections for histological examination (A, B and C) and *in situ* hybridization (D and E) showing lesions associated with the presence of the bacterium *Francisella halioticida* in mussel (*Mytilus* sp.) tissues. A. Haemocytic infiltration (arrow) in the connective tissue of the digestive gland associated with necrosis of haemocytes.

B. Undetermined bacteria (arrow) in the connective tissues of digestive diverticula (DD). C. Granulomas (asterix) in the connective tissue of the digestive gland and in epithelia of the stomach (arrow). D. Histological section of the digestive gland showing hybridization of the *F. halioticida* probes with bacteria cells (arrows) in the connective tissue of digestive diverticula (DD). E. *In situ* hybridization with *F. halioticida* probes showing labelling of bacteria in connective tissues and in gonadic follicles (G) as well as inside haemocytes (arrow).

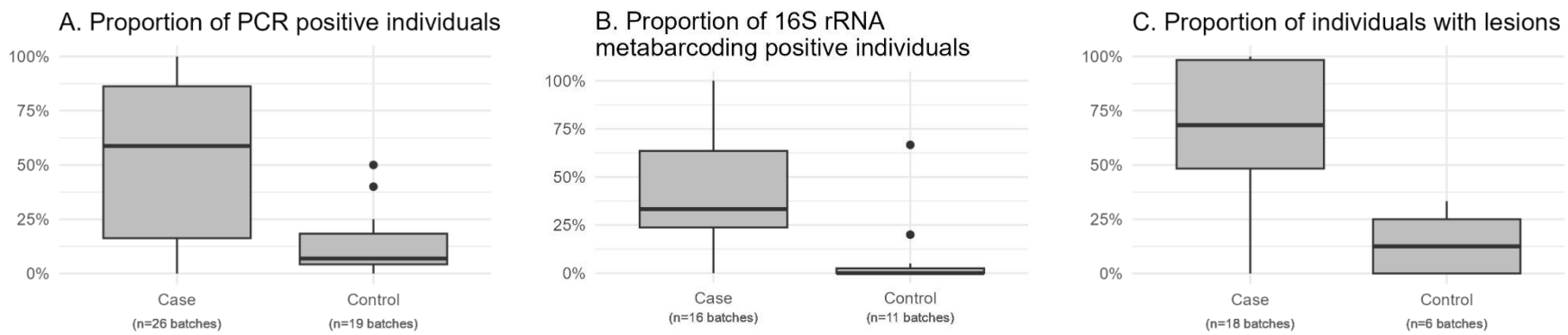

Fig.4. Distribution of proportion of positive individuals per batch (cases and controls) in which DNA of *F. halioticida* was detected: (A) by PCR or (B) 16S rRNA metabarcoding, or (C) with histological lesions. The box represents the interquartile range (lower line=first quartile, middle line = median, and upper line= third quartile), the whiskers and the dots represent respectively the variability of the minimum and maximum and the outliers in comparison the interquartile range.