High mortality of mussels in northern Brittany – Evaluation of the involvement of pathogens, pathological conditions and pollutants

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Maud Charles[1,2,*], Ismaël Bernard[3], Antonio Villalba[4,5,6], Elise Oden[2], Erika A.V. Burioli[2], Gwenaël Allain[7], Suzanne Trancart[2], Valérie Bouchart[2], Maryline Houssin[1,2]

[1] Normandie Université, Université de Caen Normandie, FRE BOREA, CNRS-7208, IRD-207, MNHN, UPMC, UCN, Esplanade de la Paix, 14032 Caen Cedex 4, France

[2] LABÉO Frank Duncombe, 1 Route de Rosel, 14053 Caen Cedex 4, France

[3] Eureka Modélisation, 22740 Lézardrieux, France

[4] Centro de Investigacións Mariñas, Consellería do Mar (CIMA), Xunta de Galicia, 36620 Vilanova de Arousa, Spain

[5] Departamento de Ciencias de la Vida, Universidad de Alcalá, 28871 Alcalá de Henares, Spain

[6] Research Centre for Experimental Marine Biology and Biotechnology (PIE), University of the Basque Country (UPV/EHU), 48620 Plentzia, Basque Country, Spain

[7] Armeria, 31B rue de la Concorde, 56670 Riantec, France

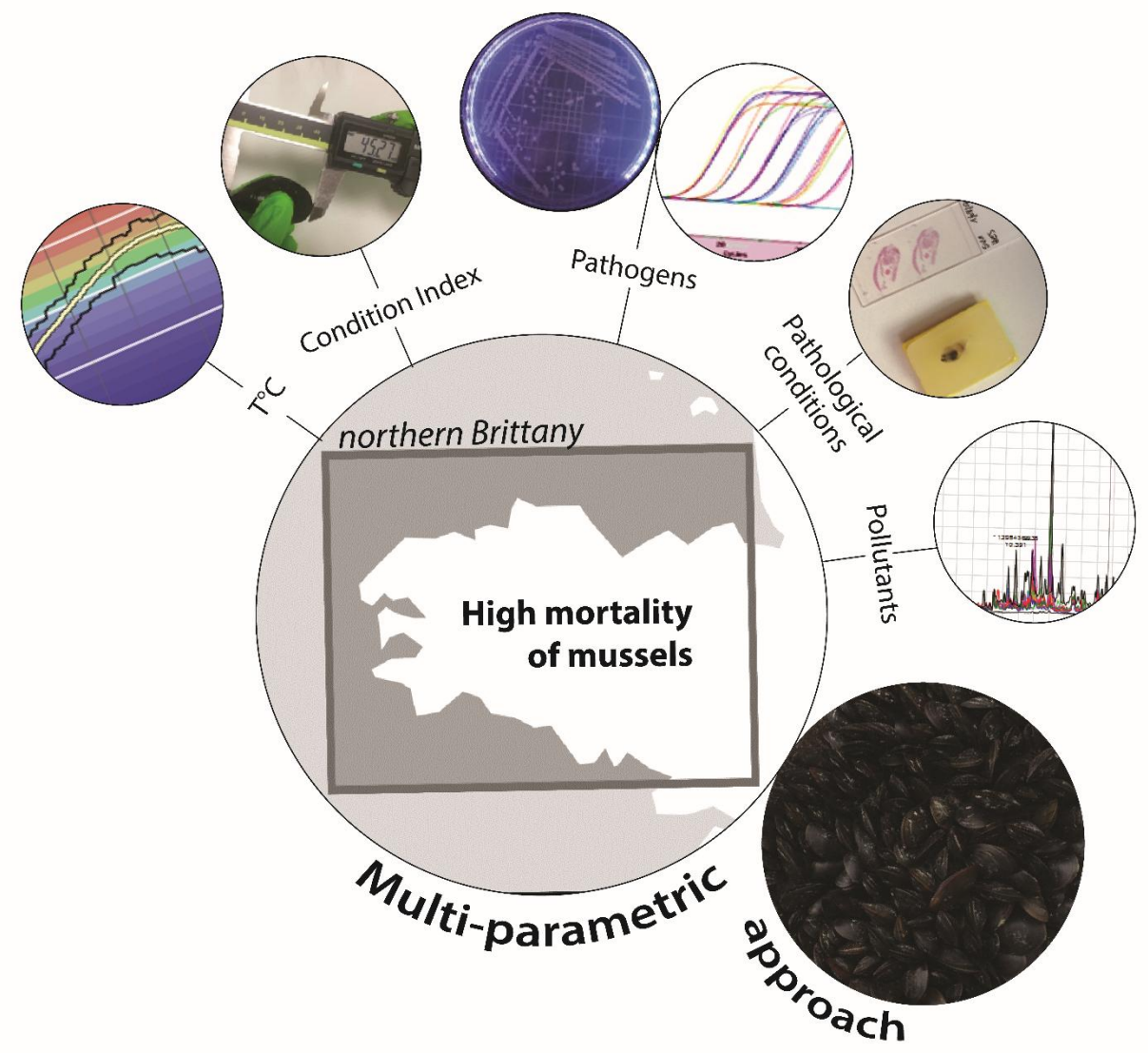
[*]Corresponding author: Maud Charles, LABÉO Frank Duncombe, Pôle Recherche, 1 Route de Rosel, 14053 Caen Cedex 4, France.

E-mail: maud.charles@outlook.fr ;

Highlights

- A multi-parametric approach was adopted to identify mortality causes in mussels.
- A ‘mortality window’ during the spring season was identified.
- Brest, Lannion and St. Brieuc (France) have different cumulative mussel mortality rates.
- Haemocytic infiltrations and *Marteilia* sp. are parameters linked to mortality.
- There was no specific significant involvement of disseminated neoplasia or *Vibrio.*

Graphical abstract



Abstract

In 2014, a high and unusual mass mortality of mussels occurred in several important production areas along the French coasts of the Atlantic and English Channel. In the first quarter of 2016, mass mortalities hit farms on the west coast of the country once again. These heterogeneous mortality events elicited a multi-parametric study conducted during the 2017 mussel season in three sites in northern Brittany (Brest, Lannion and St. Brieuc). The objective was to assess the health status of these mussels, follow mortality and attempt to identify potential causes of the abnormal high mortality of farmed mussels in northern Brittany. Brest was the most affected site with 70% cumulative mortality, then Lannion with 40% and finally St. Brieuc with a normal value of 15%. We highlighted a temporal 'mortality window' that opened throughout the spring season, and concerned the sites affected by mortality of harmful parasites (including pathogenic bacteria), neoplasia, metal contamination, and tissue alterations. Likely, the combination of all these factors leads to a weakening of mussels that can cause death.

Keywords: *Mytilus edulis*; multi-parametric; neoplasia; *Vibrio splendidus*; *Marteilia*; hemocytic infiltration

1. Introduction

Mussels play a key role in the aquatic environment and are considered to be important ecosystem engineers by providing a suitable habitat for a species rich community (Buschbaum *et al.*, 2008). Due to their intense filtration activity, they modify aquatic habitat, making it more suitable for their own species and other for organisms (Borthagaray & Carranza, 2007). Mussels are also an important food source for many aquatic and terrestrial animals including humans, making them a product with high economic value. The blue mussel industry is important in Europe, a main producer of mussels globally, and France is second in Europe with a production of around 80,000 tons per year, most from aquaculture (FAO, 2018). In France, Brittany is a commercially important area for shellfish farming and is the second mussel producing region with 17,500 tons of *Mytilus* spp. produced in 2016 (CNC, 2016).

In 2014, France was affected by high and unusual mass mortality of mussels, both juveniles and adults, affecting several important production areas along the French coast of the Atlantic and English Channel. Production losses reached 50 - 100% depending on the sites (Béchemin *et al.*, 2015; François *et al*., 2015; Allain & Bernard, 2016; Lupo *et al.*, 2016).The phenomenon again affected farms on the west coast of France in the first quarter of 2016 (FAO, 2016). Unlike mortality events in Pacific oyster (*Crassostrea gigas*), very few massive moralities of *Mytilus* spp. occurred along the French coast until the winter of 2014 (Brienne, 1964; Blateau, 1989; Guichard *et al*., 2011).

One of the proposed causes of mortality is pathogenic bacteria in the genus *Vibrio* (Béchemin *et al*., 2015). Vibrios are among the major bacterial pathogens of marine organisms and have been identified as pathogens of several bivalve molluscs (Beaz-Hidalgo *et al*., 2010; Lemire *et al*., 2015; Eggermont *et al*., 2017). During mussel mortality events, different *Vibrio splendidus* strains were isolated from moribund mussels in France and were linked to mortality (Travers *et al*., 2015; Ben Cheikh *et al*., 2016). Of the sites studied, the Bay of

Brest, located at the extremity of the Brittany Peninsula, is one of the locations where mussels suffered massive mortality in 2014 and beyond. Estimated losses were 30% to 80%, depending on the year. *Vibrio* strains and pathogenic bacteria belonging to the Splendidus clade were identified and associated with these mortalities (François *et al*., 2015; Lupo *et al.*, 2016). Other researchers have suggested that the cause of mussel mortalities is disseminated neoplasia. This disease is well documented in marine bivalves all over the world (Peters, 1988; Elston *et al.*, 1992; Landsberg, 1996; Carballal *et al*., 2015) and, in some bivalve species including the mussel *Mytilus trossulus*, spreads by transmission of cancerous cells between individuals (Metzger *et al.*, 2015, 2016). Some scientists observed chromosomal abnormalities associated with neoplasia in *Mytilus trossulus* (González-Tizón *et al*., 2000) while advanced neoplastic disease was associated with significant mortalities of *Mytilus* sp. (Moore *et al*., 1991), including *M. trossulus* in Washington and Oregon (USA) and in British Columbia (Canada) (Elston *et al.*, 1992). In France, Benabdelmouna & Ledu (2016) and Benabdelmouna *et al*. (2018) presented evidence of the involvement of genomic abnormalities in mortality outbreaks in blue mussels and linked them to disseminated neoplasia. In addition, some cases of disseminated neoplasia in *Mytilus edulis* from Lannion (northern Brittany, France) were observed in mid-October 2016 by Burioli *et al.* (2017) during a mortality event.

Following heterogeneous mortality events of mussels that occurred in Brittany in the past years (Allain & Bernard, 2016; Bernard & Allain, 2017), the Regional Shellfish Committee of North Brittany (CRCBN) set up mortality monitoring in 2017 with coupled samplings over a period of 8 months. The initial focus was on the detection of neoplasia and on the involvement of a specific strain of *V. splendidus*. Nevertheless, it is known that the magnitude of mortality in bivalves can be modulated by several biotic factors, including the presence of pathogens (Carrasco *et al*., 2015), and by abiotic factors such as chemical contaminants (Coles *et al.*, 1995; Pipe & Coles, 1995; Dailianis, 2010; Moschino *et al.*, 2016), water

temperature and animal conditioning (Almada-Villela *et al*., 1982; Seed & Suchanek, 1992; Gosling, 1992, 2003). In an exploratory process, a field study conducted in partnership with mussel farmers was conducted *in situ,* wherein analyses, including biometry (condition index), histopathological examination, determination of bacterial flora, detection of bivalve mollusc pathogens and measurement of trace elements in mussel tissues, were carried out in three different sites in northern Brittany. Our objective of this *in situ* study was to identify potential causes responsible for the abnormal high mortality of farmed mussels in northern Brittany.

2. Materials and methods

2.1. Sampling sites and mussels

Three primary mussel production sites in northern Brittany were selected for this study: the bays of Brest (Finistère, France), Lannion (Côtes-d'Armor, France) and St. Brieuc (Côtes-d'Armor, France) (Fig. 1). In each bay, one mussel farming company was involved in the study, representing approximately 8% of the mussel farmers in the Bay of St. Brieuc, approximately 25% of those located in the Bay of Brest, and the only farmer working in the Bay of Lannion. In each site, mussel farmers had introduced 7-mo-old mussels (23.51 ± 4.62 mm in length) in the fall 2016 (Sept./Oct.) for their 2017 production season. These mussels were used in this study and were monitored until September 2017. The mussels of Brest originated in La Plaine-sur-mer (Loire-Atlantique, France), those of Lannion in Pénestin (Morbihan, France) and those of St. Brieuc in Noirmoutier (Vendée, France). The maximum distance between the origination sites is 60 km. The aim of this field study was to monitor and analyse mussels that were representative of those cultivated by the mussel farmers in their area, which depended exclusively on where mussel farmers obtained the spat.

The identification of species was determined from 400 mussels (50 per date and per site) sampled only for this purpose (Table 1). The determination of the species was performed by genotyping on a set of ancestry informative SNPs (Simon *et al.,* 2018).

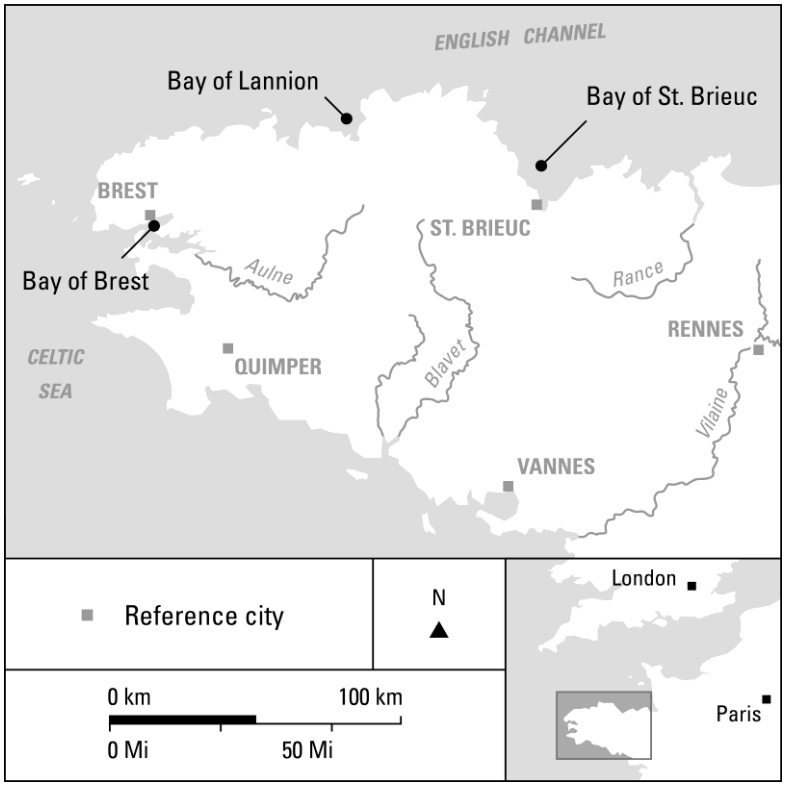


Figure 1: Location of the three sampling sites: the bays of Brest, Lannion and St. Brieuc.

2.2. Mortality and temperature monitoring

In each site, 30 baskets of 100 mussels were prepared (Fig. 2A) and fixed on intertidal mussel stakes ("*bouchot"*; Fig. 2B) or subsurface longlines (for Lannion) at the beginning of the study. Every month, two baskets per site were collected and live mussels were counted for mortality monitoring. For Brest and St. Brieuc, in order to limit the time dedicated to counting during the tide, different baskets were counted each time; while for Lannion, because the tide is not a constraining factor, the same mussels were counted and put back into the baskets. Thus, each month, 200 mussels per site were counted to estimate mortality. Temperature sensors (HOBO Pendant®) were placed in the baskets containing mussels. These recorded the temperature around the animals (either water temperature or air temperature depending on tide) every 30 min.

Figure 2: Pictures of (A) the preparation of the baskets each containing 100 mussels and (B) the fixing of the baskets on the intertidal mussel stakes ("bouchot"). Photo credit: Regional Shellfish Committee of North Brittany (CRCBN)

2.3. Sampling for analysis

Sampling for analysis was carried out with different frequency among sites: February, April, May and September 2017 for Brest, and February and May 2017 for Lannion and St. Brieuc (Table 1). At each sampling, 50 mussels per site were collected from baskets for histology, and 20 additional mussels for biometry, molecular biology, bacteriological and chemical analyses (performed on the same animals). The collected mussels were transported in thermal bags to the laboratory and either analysed immediately or held in a refrigerator (4 °C) overnight and analysed the next day.

Table 1. Information on study sites, sampling dates and number of mussels used for each analysis.

Site	Latitude	Longitude	Farming method	Sampling date				Species identification	Histological examination	Other analyses (biometry, bacteriological, molecular, and chemical analyses)
				Feb.	Apr.	May	Sept.			
Bay of Brest	48°20'22.5"N	4°19'48.4"W	*Bouchot*	27/02/2017	24/04/2017	30/05/2017	20/09/2017	50 per date (total=200)	50 per date (total=200)	20 per date (total=80)
Bay of Lannion	48°44'44.8"N	3°35'24.9"W	Longlines	24/02/2017		17/05/2017		50 per date (total=100)	50 per date (total=100)	20 per date (total=40)
Bay of St. Brieuc	48°33'07.6"N	2°38'55.6"W	*Bouchot*	27/02/2017		28/05/2017		50 per date (total=100)	50 per date (total=100)	20 per date (total=40)

2.4. Biometry

All mussel samples were individually weighed (wet flesh and shell separately after drainage of intervalvar water) with a precision scale (± 0.01 g). A condition index (C.I.) was calculated as follows (Davenport & Chen, 1987):

$$C.I. = \frac{Wet\ meat\ weight}{Shell\ weight}\ X\ 100$$

2.5. Histological examination

For dissection, shells were opened by cutting the adductor muscle and the soft tissues were removed. An approximately 5 mm thick transverse section of mussel tissue containing mantle lobes, visceral mass (gut, digestive gland) and gills was excised, placed into histological cassettes and transferred to Davidson's fixative for 48 h before being transferred to 70% ethanol. Fixed tissues were then dehydrated through an ascending ethanol series and embedded in paraffin wax. Thick sections, 5 µm, were obtained using a rotary microtome and then stained with Harris' hematoxylin and eosin (HHE) (Howard *et al.*, 2004). Histological sections were examined for all symbionts, including phoresis, commensalism, parasitism and mutualism (Kinne, 1980), and pathological conditions under light microscopy. For each sampling date indicated in Table 1, 50 histological slides corresponding to the 50 sampled mussels were examined.

2.6. Bacteriological analysis

Analysis was conducted to compare the bacterial flora of mussels from each of the three sites on two different dates (February and May). For each of the six batches, tissues of five live mussels (to avoid the emergence and overexpression of opportunistic bacteria due to tissue degradation in dead animals) were pooled and mashed with scalpels and 200 µL of Artificial Sterilized Sea Water (ASSW) was added to 50 mg of the homogenate. After stirring and pulse centrifugation to pellet cellular debris, ten-fold dilutions of supernatant were made and 100

µL of 1:100 and 1:10,000 dilutions were sown on Zobell marine agar (Agar and ASSW enriched with 0.5% peptone, 0.1% yeast extract, and 0.01% ferric phosphate, pH 7.6; Oppenheimer & ZoBell, 1952) and incubated for 48 h at 22 °C. For each pool, 20 predominant bacterial colonies with different phenotypes were re-isolated in order to verify their purity. DNA extraction was performed by heating a colony placed in 250 µL of purified water (DNA/nuclease free-water) for 10 min at 95 °C. Successful extraction was confirmed by DNA quantification with NanoDrop™ 2000c spectrophotometer (ThermoFisher Scientific™, Waltham, MA USA). Then, strains were characterized by molecular analysis (see below *2.6.1*.).

2.7. Molecular analyses

The first step to determine the bacterial profile of the mussel batches consisted in discriminating between strains related to the *V. splendidus* clade (*V. splendidus*-related species) and other marine bacteria, as some vibrio strains belonging to this Splendidus clade are known to be pathogenic for molluscs. One TaqMan® real-time PCR, targeting the *16S rRNA* gene of *V. splendidus*-related strains (PCR1) (Oden *et al*., 2016), was carried out on a Smart Cycler® (Cepheid, USA); the primers used for PCR1 were *SpF1* 5'ATCATGGCTCAGATTGAACG3' and *SpR1* 5'CAATGGTTATCCCCCACATC3' (Nasfi *et al*., 2015) and the probe *SpProbe* 5'CCCATTAACGCACCCGAAGGATTG3'. The reaction volume of 25 µL contained 12.5 µL of Premix Ex Taq® 2 X Takara® (Lonza, Verviers, Belgium), 0.5 µL of each primer (20µM), 0.5 µL of probe (10 µM), 9 µL of purified water and 2 µL of extracted DNA (replaced with 2 µL of purified water in the negative control). The thermal cycling profile was 95°C for 10 s, followed by 40 cycles at 95 °C for 5 s and 62 °C for 30 s. When PCR1 was positive, a conventional PCR targeting the housekeeping genes *mreB* of *V. splendidus*-related strains (PCR2) was performed because it is the most discriminant gene for the identification of closely related strains among the Splendidus clade

(Oden *et al.*, 2016). The primer pair for PCR2 was *mreB*-F 5'CTGGTGCTCGYGAGGTTTAC3' and *mreB*-R 5'CCRTTYTCTGAKATATCAGAAGC3′. For colonies not identified as members of the Splendidus clade (PCR1 negative), another conventional PCR targeting the *16S rRNA* gene (PCR3) (Burioli *et al.*, 2018) was done; the primer pair for PCR3 was *16S27*-F 5'AGAGTTTGATCMTGGCTCAG3' and *16S1492*-R 5'ACCTTGTTACGACTTCAC3'. For the conventional PCR, typical 25-µL reaction mixtures contained 12.5 µL of Premix Ex Taq® 2 X Takara® (Lonza, Verviers, Belgium), 0.5 µL of each primer (20 µM), 9.5 µL of purified water and 2 µL of DNA template (replaced with 2 µL of purified water in the negative control). Conventional PCR amplifications were carried out in a T100™ Thermal Cycler (Bio-Rad, France) and the thermal program was as follows: 10 s at 95°C; 30 cycles for 10 s at 95°C, 30 s at 55°C, 40 s at 72°C and a final extension of 3 min at 72°C. PCR products were then analysed with QIAxcel® Advanced System (Qiagen, Courtaboeuf, France) and those with the expected size were sent to Eurofins MWG Operon (Ebersberg, Germany) to be purified and sequenced. Species were identified using the National Center for Biotechnology Information (NCBI) Basic Local Alignement Search Tool (BLAST) with individual *16S rRNA* sequences, and *mreB* sequences were aligned using a multiple sequence alignment Multiple Sequence Comparison by Log-Expectation (MUSCLE). Phylogenetic analyses were performed in MEGA7 (Kumar *et al.*,, 2016) using the Neighbor Joining method (Tamura *et al*, 2013) and the maximum composite likelihood model with a bootstrap of 1000 replications; sequences of the *mreB* gene from 44 different reference bacterial strains from the Splendidus clade were used (see *2.1.* in Oden *et al*., 2016). *V. aestuarianus* 02/041, *V. ordalii* 12B09 and *V. penaeicida* AQ115, were provided from the Genomic of *Vibrio* Research Department (CNRS Roscoff, France) and used as the Splendidus clade outgroup.

In parallel, the presence of known bivalve pathogens (*Bonamia* spp., *Haplosporidium nelsoni*, *Marteilia* sp., *Mikrocytos mackini*, *Nocardia crassostreae*, Ostreid Herpesvirus type 1

(OsHV-1), *V. aestuarianus*, *V. tubiashii*, *V. harveyi*, and *V. splendidus*) was investigated by PCR on DNA extracts from mussel tissues (Additional file 1).

2.8. Chemical analyses of flesh

The objective of chemical analyses was to assess if one site was more contaminated than others by one or more chemical compounds, which might explain why mortality was higher in one than in the others. For cost reasons, there were limitations in the number of samples that could be analysed. Because mortality began to increase in February, we chose to analyse and compare the three sites during that month rather than in May.

Presence of trace metal compounds (Pb, Hg, Cd, Co, Mo, Sn, Cr, Cu, Ni, Zn, Fe, Al, Mn and Ti) and non-metallic trace elements (As and Se) in mussel flesh was quantitatively determined by inductively coupled plasma-mass spectrometry (ICP-MS Triple Quad 8800, Agilent Technologies, Santa Clara, CA, USA) after wet digestion through high-quality grade mineral acids (HNO_3, Analpure®, Analytika® , Prague, Czech Republic) and oxidants (H2O2, Normapur®,VWR international, Radnor, Pennsylvanie, USA), following previously described protocols (Squadrone *et al*., 2016). All analyses were calibrated against analytical standards. The limit of quantitation was 0.02 mg/kg for elements analysed by ICP-MS. All concentrations are given on a wet weight (w. w.) basis.

2.9. Data analysis

2.9.1. Half-stock index

To compare mortality phenomena over time, the half-stock index (Bernard *et al*., 2018) is more suitable than using a cumulative mortality rate since it allows visualization of what is happening at a given time. Indeed, to compare different mortality episodes, it is preferable to consider the exponential aspect of population decrease. Thus, if λ is the mortality rate, supposed constant $N(t)$ the population at t time, and N_0 the population at the beginning of the

study, then: $N(t) = N_0^{e- \lambda t}$. Between two successive counts in t_{i-1} and t_i, there is only one λ which describes these two points:

$$\lambda = \frac{ln\,(N_{i-1}) - ln\,(N_i)}{t_i - t_{i-1}}$$

To obtain a more tangible index, the time (in days) needed to halve the population was used: $d_{0.5} = ln\,(2) / \lambda$. Therefore, this index corresponds to the number of days it takes to halve the population with the measured mortality rate, in a very similar way to half-life of radioactive elements. The lower this index, the higher the mortality rate and decrease of the stock. In the absence of mortality, this index tends towards infinity. For a better graphical representation, a maximum value was assigned to this index equal to 1500 (which corresponds to a loss of 15% over 1 year).

2.9.2 Statistical analysis

The half-stock index has been used to analyse and compare instantaneous mortality with different sampling intervals. It has also been used as the variable response to link the variation of mortality with the prevalence of pathological observations. This relationship was tested using a Spearman correlation test. Temperatures presented here have been smoothed in order to represent annual variation using generalized additive models with smoothing term with a moving window corresponding to one month.

Regarding biometry data, replicates were averaged, and the values were tested for normality (Shapiro). Then, a first ANOVA was used to compare the C.I. of Brest mussels at the four sampling dates, and a second to compare the C.I. of mussels from the three sites on both dates (February and March). When a significant difference was obtained, paired comparisons by Tukey's *post hoc* test were performed to identify which C.I. were different from the others. Statistical significance was accepted for $*p < 0.05$. The association between the C.I. and observed mortality was evaluated using a Pearson correlation test. Statistical analyses and graphical representations were performed by using R software, version 3.5.1 (https://www.r-project.org/) and 'dplyr', 'tidyr' and 'ggplot2' packages.

3. Results

3.1. Mussel species, temperature monitoring and mortality

For species identification, a dataset of 81 common markers was obtained (for more details, see Simon *et al*, 2019), which allowed identification of all mussels as the species *M. edulis*.

Regarding thermal profiles (Fig. 3), Lannion appears to be a rather temperate site compared to those of Brest and St. Brieuc, which showed wider temperature variation, especially higher temperatures in summer. From February to August, the temperature of Lannion rose from 9 to 18 °C, that of St. Brieuc from 9 to 20 °C and that of Brest from 8.5 to 20 °C.

The first abnormal mortalities were observed at the very end of winter and early spring 2017 (Fig. 3) in the most western site. Indeed, Brest was the most affected site as it suffered continuous mortality as illustrated by the half-stock index until mid-summer (Fig. 3B**)**. The site had nearly 70% cumulative mortality at the end of the study (Fig. 3A**)**. Lannion was less severely affected than Brest –but more than St. Brieuc– with a progressive decreasing trend in the half-stock index leading to a final cumulative mortality of almost 40%. St. Brieuc experienced some sporadic mortality peaks, but not considered abnormal. At the end of the study, a cumulative mortality of about 15% was obtained, which was a typical value for institutions (Council Directive 95/70/EC abrogated by 2006/88/CE) and mussel farmers. Furthermore, mortality decreased significantly or even stopped in mid-summer when temperatures exceeded 18 °C.

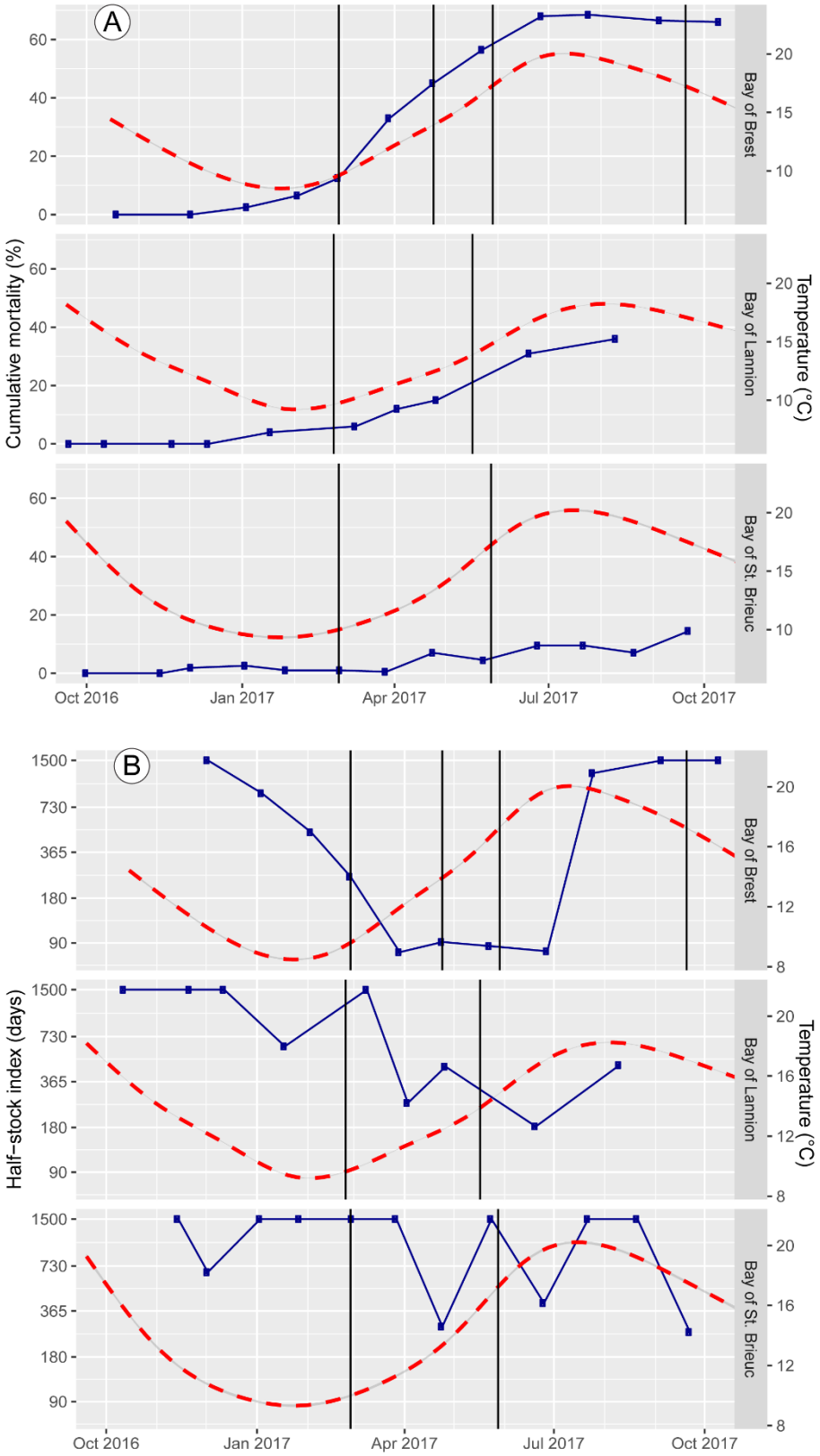


Figure 3: Variation of temperature (broken line) and: (A) cumulative mortality (%) observed over the duration of the study based on monthly mortality monitoring (continuous line with dots); (B) half-stock indices (days) calculated monthly to visualize the occurrence of mortalities for each month (line with dots). The vertical black lines correspond to the dates of sampling for analyses.

3.2. Biometry

When the C.I. of Brest mussels at the four sampling dates between February and September 2017 were compared, despite an upward trend in the April index, no significant difference was observed (ANOVA, $p > 0.05$) (Fig. 4). When the C.I. of mussels from the three sites on both dates (February and May 2017) were compared, no significant differences was observed in February but in May, the C.I. of mussels from Lannion were significantly higher than those of Brest mussels (ANOVA, $p < 0.05$ and Tukey's *post hoc* test) (Fig. 5). There was no significant difference between the two C.I. in February and in May for the same site and no significant link appeared between observed mortality and C.I. (Pearson correlation, $p > 0.05$).

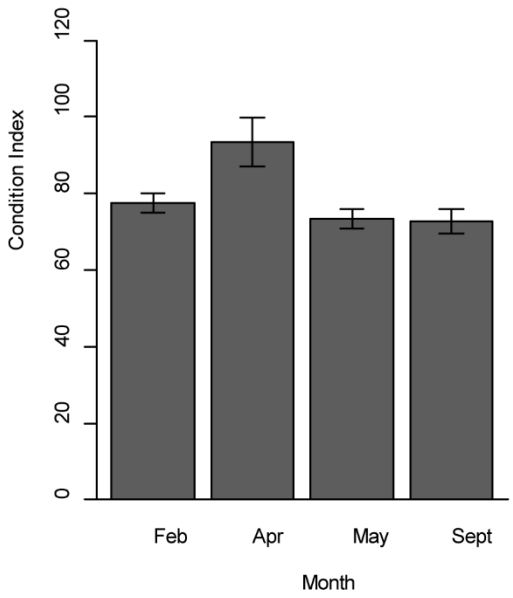


Figure 4: Evolution of the Condition Index (C.I.) of the Brest mussels on four dates (February, April, May and September 2017). Values are means ± SEM (ANOVA, $p > 0.05$).

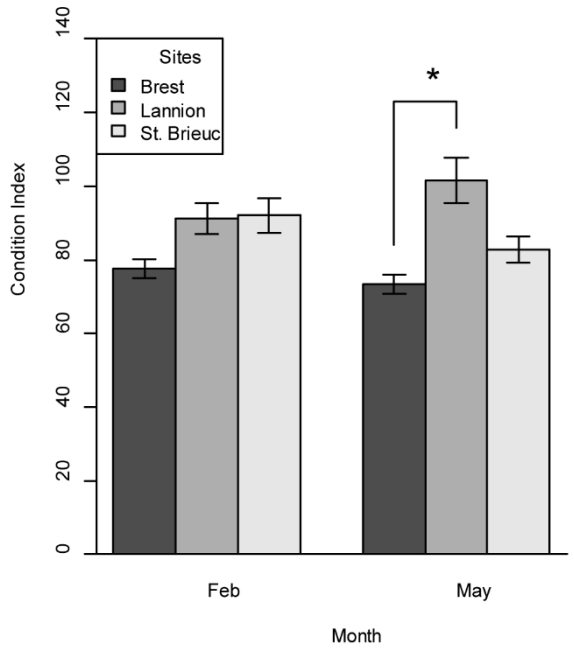


Figure 5: Comparison of the Condition Indices (C.I.) of mussels from the 3 sites (Brest, Lannion, St. Brieuc) on both dates (February and May 2017). Values are means ± SEM (ANOVA, *p < 0.05 and Tukey's *post hoc* test).

3.3. Histological examination

Histopathological examination showed the occurrence of various symbionts and pathological conditions. Regarding symbionts, the different stages characterising parasites of the genus *Marteilia* were observed in the stomach and digestive diverticula epithelia (Fig. 6A), from primary cells enclosing some secondary cells in the stomach and digestive diverticula epithelium to fully developed stages in digestive diverticula, in which secondary cells included tertiary cells and had refringent granules in their cytoplasms. Occasionally, early stages of *Marteilia* sp. were observed in the gills, associated with heavy inflammatory response. Two types of ciliates were also observed, one type resembling *Ancistrum mytili* in the gills (Fig. 6B) and intracellular ciliates in the epithelium of digestive tubules (Fig. 6C). Trematode sporocysts enclosing developing cercariae were observed in the connective tissue of the mantle and the visceral mass (Fig. 6D). *Mytilicola* sp. copepods were found in the intestinal lumen (Fig. 6E). Regarding other pathological conditions, cases of heavy hemocytic infiltration of the connective tissue of different organs were detected, but without any identifiable inducing agent (Fig. 7A). The occurrence of large masses of hemocytes, mostly

granulocytes, surrounded by several layers of flattened, epithelioid cells, were also observed in the connective tissue of various organs (Fig. 7B). This type of inflammatory structure is usually called a granulocytoma and its occurrence was not linked to any detectable agent. Finally, cases of disseminated neoplasia, characterised by the infiltration of the connective tissue by abnormally large cells that also proliferate through the circulatory system (Fig. 7C) were observed. The nucleus of the abnormal cells was significantly larger than that of normal cells, with at least one patent nucleolus, showing frequent mitotic figures that indicated a high division ratio (Fig. 7D). The prevalence of symbionts and pathological conditions in each site is shown in Fig. 8. Regarding those with potentially the most serious consequences (granulocytomas, hemocytic infiltration, disseminated neoplasia, trematode sporocysts and *Marteilia*), Brest was the most affected site (Fig. 8A). Indeed, it was the only site in which 4 out of 5 serious pathological conditions occurred in February (granulocytomas: 2.9%; hemocytic infiltration: 14.3%; trematode sporocysts: 11.4% and *Marteilia*: 22.9%) and in May (granulocytomas: 4.1%; hemocytic infiltration: 26.5%; trematode sporocysts: 2.0% and *Marteilia*: 22.5%). It was also the only site affected with *Marteilia* sp., which occurred over the entire study period and had a prevalence peak in April with 36% of individuals infected (Fig. 8B). In February, Lannion and St. Brieuc were affected by granulocytomas with similar prevalence (6.7% and 6.5%, respectively), twice that of Brest, and twice as many individuals showed trematode sporocysts at St. Brieuc (6.5%) than at Lannion (3.3%). In May, a low percentage of Lannion mussels had disseminated neoplasia (2.4%), while it was not detected in St. Brieuc. It is important to note that St. Brieuc had no sign of hemocytic infiltration, neither in February nor May. In contrast, St. Brieuc was the most affected by two less damaging symbionts, ciliates and *Mytilicola* sp., both in February and May. The prevalence of hemocytic infiltration increased in Brest from February to May, while it remained almost constant at Lannion. Figure 8B illustrated these observations for Brest; indeed, there was an increase in hemocytic infiltration and *Marteilia* between February and April, then a gradual

decrease from April to September. Furthermore, 18% of mussels from Brest showed disseminated neoplasia in April, while it was not found in February or May, and its prevalence was 2% in September. In Brest, the prevalence of most symbionts and the other pathological were highest in April. The prevalence of the symbionts and other pathological conditions showed an inverse relationship with the half-stock index. When testing the link between the prevalence of the different pathological conditions and the half-stock index (Spearman correlation test; Fig. 9), only hemocytic infiltration was significantly positively correlated to the intensity of mortality ($p^* < 0.05$). Nevertheless, the p-value of *Marteilia* – equal to 0.08– was influenced by its absence in Lannion and St. Brieuc; in Brest, the higher the prevalence of *Marteilia*, the lower the half-stock index.

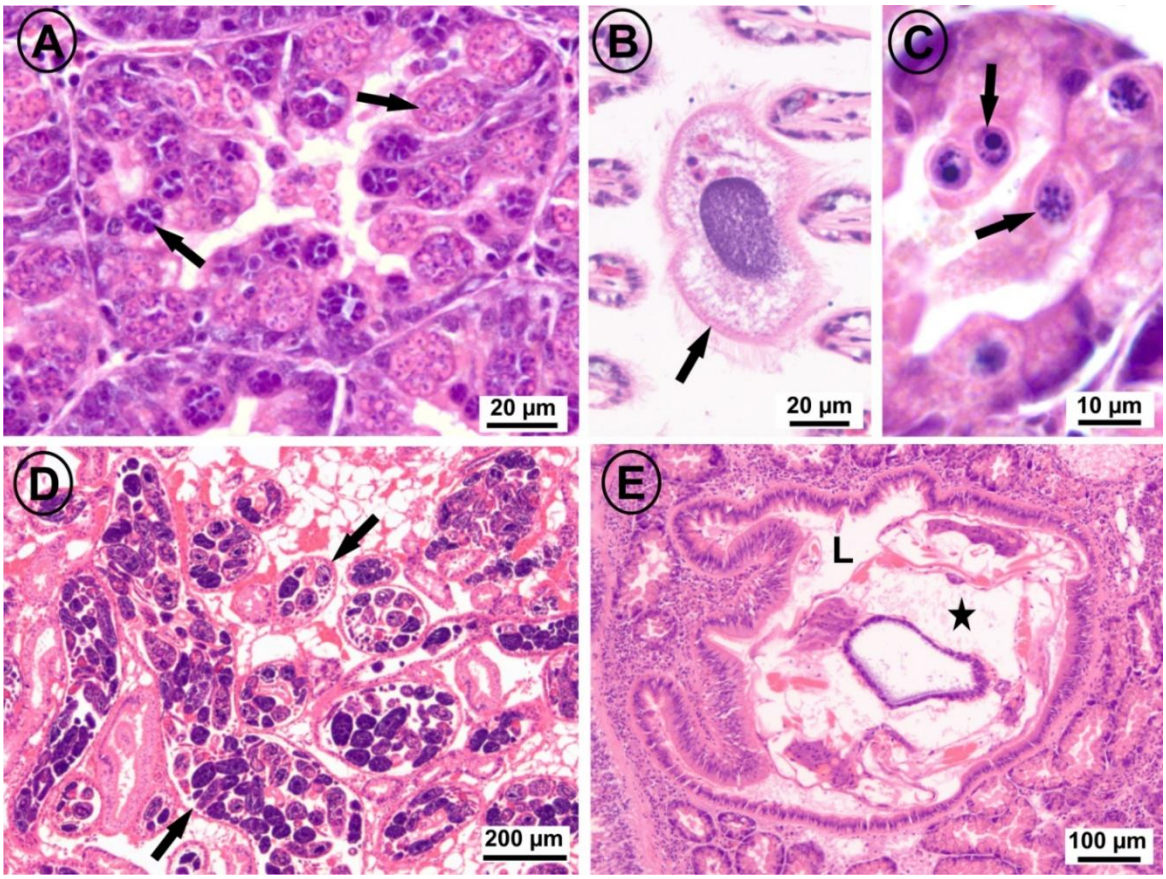


Figure 6: Micrographs of histological sections showing symbionts found in *Mytilus edulis*. A: section of the digestive gland showing different developing stages of *Marteilia* sp. (arrows) in the epithelium of digestive tubules. B: section through the gills showing a cell of the ciliate *Ancistrum mytili* (arrow). C: section through the digestive gland showing various intracytoplasmic ciliates (arrows) in the epithelium of a digestive tubule. D: section through the mantle showing trematode sporocysts (arrows) enclosing cercariae. E: section through the visceral mass showing a copepod *Mytilicola* sp. (star) in the intestinal lumen (L).

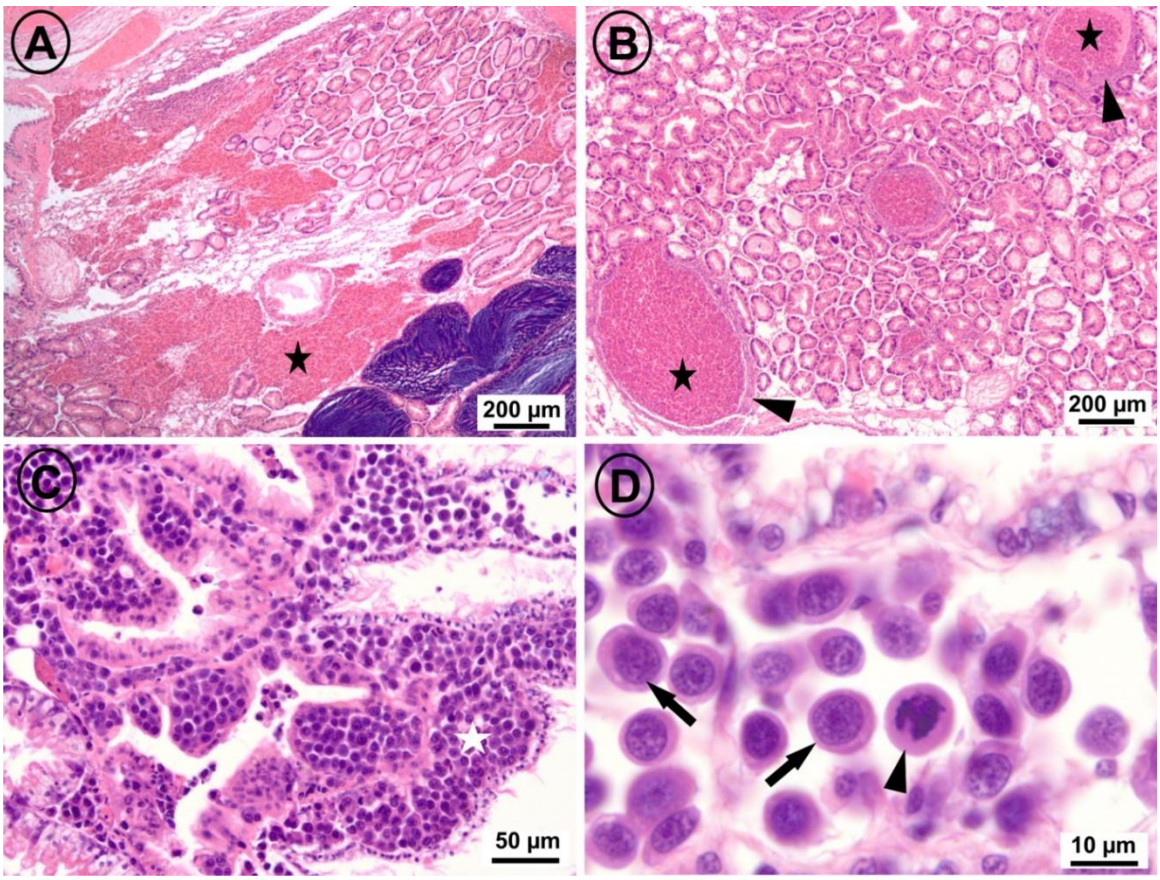


Figure 7: Micrographs of histological sections showing pathological conditions of *Mytilus edulis*. A: section through the visceral mass showing heavy hemocytic infiltration (star) of the connective tissue. B: section through the digestive gland showing granulocytomas consisting of large masses of hemocytes (stars) surrounded by layers of flattened, epithelioid cells (arrowheads). C: section through the visceral mass showing the connective tissue heavily infiltrated with masses of neoplastic cells (star). D: Higher magnification of the previous micrograph showing abundant neoplastic cells (arrows) in a hemolymph sinus: a cell in mitotic process is pointed out with an arrowhead.

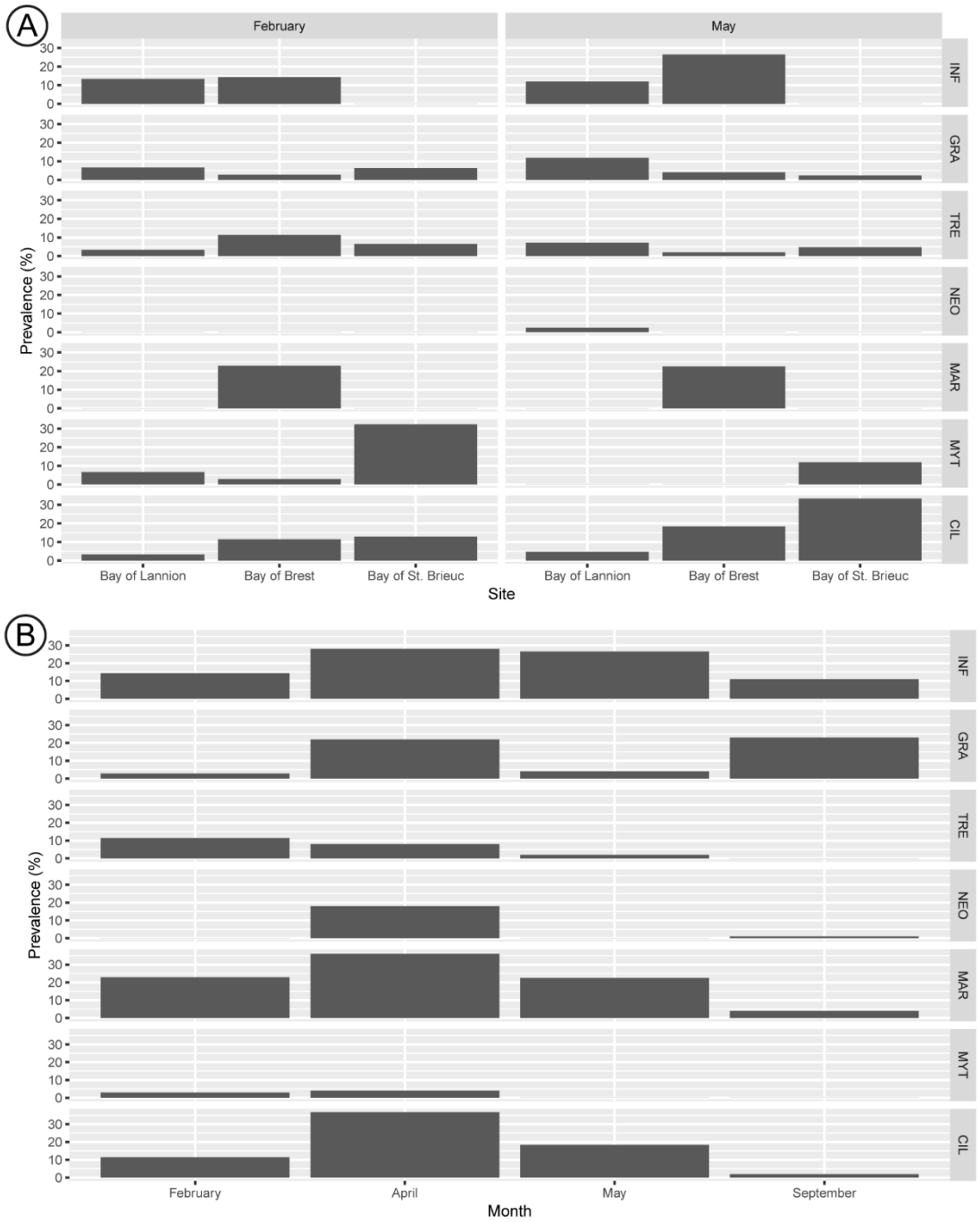


Figure 8: Prevalence (%) of the most remarkable histopathological observations: granulocytomas (GRA), inflammation/haemocytic infiltration (INF), disseminated neoplasia (NEO), trematode sporocysts enclosing cercariae (TRE), *Marteilia* sp. (MAR), *Mytilicol*a sp.(MYT) and ciliates (CIL) identified (A) in the 3 bays (Brest, Lannion, St. Brieuc) on both dates (February and May 2017); (B) in Brest on four dates (February, April, May and September 2017).

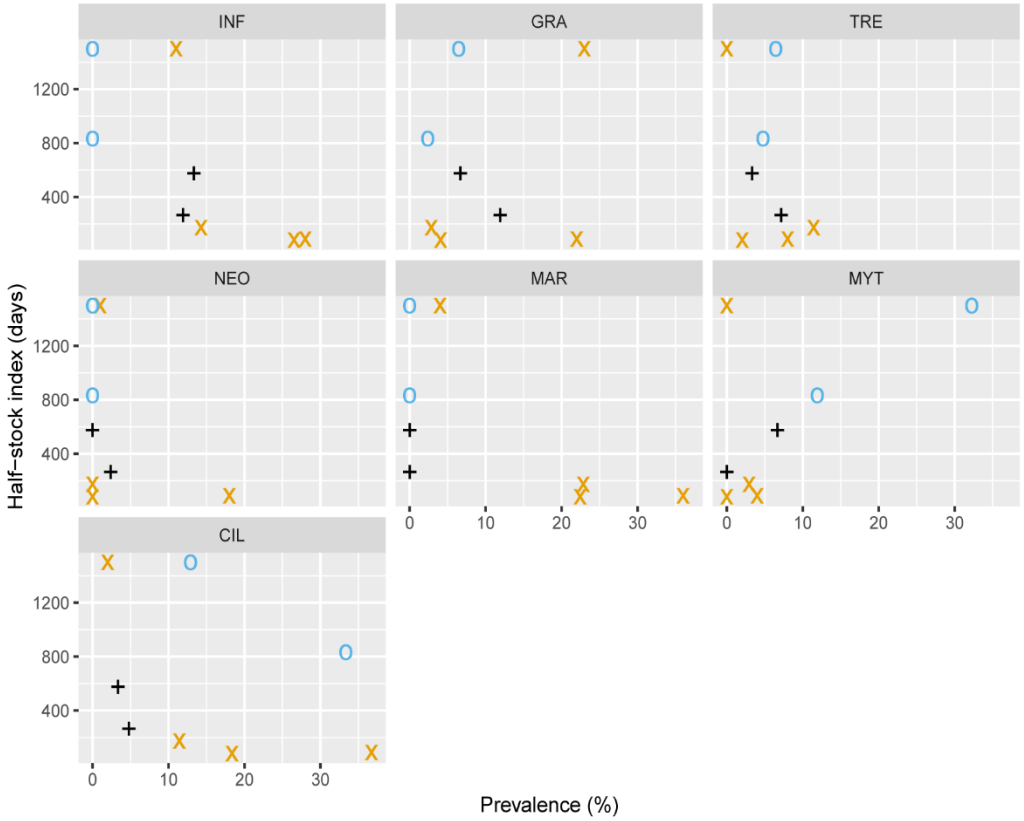


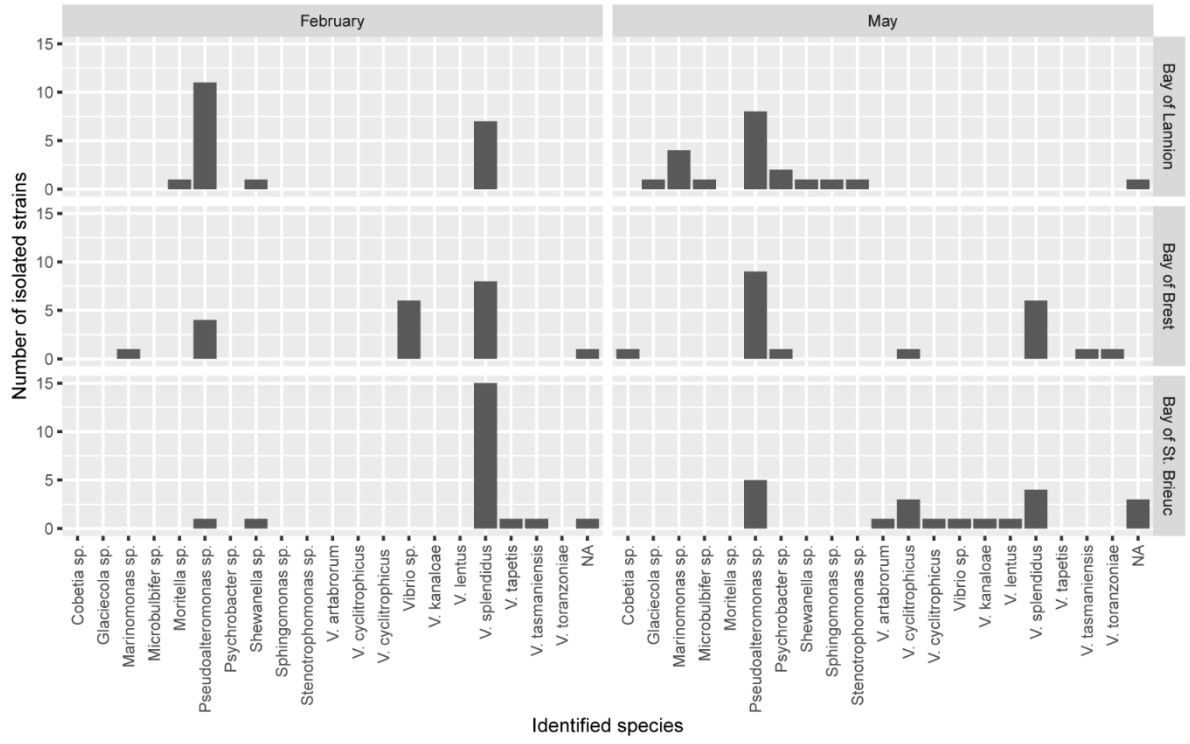
Figure 9: Link between the prevalence (%) of symbionts and histopathological conditions and the half-stock index (days) for each site: Brest (letter x), Lannion (plus sign) and St. Brieuc (open circle). INF: haemocytic infiltration; GRA: granulocytomas; TRE: trematode sporocysts enclosing cercariae; NEO: disseminated neoplasia; MAR: *Marteilia* sp.; MYT: *Mytilicol*a sp.; CIL: ciliates.

3.4. Molecular analyses

3.4.1. Bacterial profiles

Following the identification of the 120 isolated bacterial strains from tissue culture, it was noted that bacterial diversity was relatively higher in May than in February (Fig. 10). In February, between four (Brest & Lannion) and five (St. Brieuc) different species per site were present among the 20 strains isolated compared to seven (Brest & St. Brieuc) and nine (Lannion) different species in May. However, despite this difference in diversity, two bacteria remained dominant: *V. splendidus* and *Pseudoalteromonas* sp. *V. splendidus* prevalence in February was 42% in Brest, 35% in Lannion and 80% in St. Brieuc; in May, prevalence was 30% in Brest, 0% in Lannion and 23% in St. Brieuc. In February *Pseudoalteromonas* sp. prevalence was 21% in Brest, 55% in Lannion and 5% in St. Brieuc; while in May, prevalence was 45% in Brest, 42% in Lannion and 29% in St. Brieuc. Among the 30 strains of

263 *V. splendidus* isolated in the three February batches –7 in Lannion, 15 in St. Brieuc and 8 in
264 Brest– all had a different *mreB* sequence, except for three strains found in St. Brieuc (1) and
265 in Brest (2), which had an identical sequence. When comparing bacterial profiles with the
266 occurrence of mortalities, no difference appeared; *V. splendidus* was found in high abundance
267 in almost all sites, as was *Pseudoalteromonas* sp. to a lesser extent, with or without
268 mortalities.
269



270

271 Figure 10: Bacterial profiles in samples from the 3 bays (Lannion, Brest, St. Brieuc) collected in February and
272 May 2017.
273

274 *3.4.2. Presence or absence of known pathogens of bivalve molluscs*

275 Among the four pools of DNA extracts from tissues of each of the nine batches we
276 investigated presence of 10 known pathogens, *Bonamia* spp., *Haplosporidium nelsoni*,
277 *Marteilia* sp., *Mikrocytos mackini*, *Nocardia crassostreae*, OsHV-1, *V. aestuarianus*, *V.*
278 *tubiashii*, *V. harveyi*, and *V. splendidus* belonging to Splendidus cluster. *V. splendidus* was
279 detected in all the batches from each site, while *Marteilia* sp. was recorded in all batches from

Brest (from February to September), which confirmed the histological results. All the other TaqMan® real-time PCR results were negative.

3.5. Chemical analyses of flesh

Table 2 shows trace element concentrations in the mussel tissues and the maximum level (ML) set by the Commission Regulation ((EC) No. 1881/2006 amended by (EC) No. 629/2008 and (EC) No. 420/2011) for regulated chemical compounds in the relevant foodstuffs category of bivalve molluscs. Comparing the three sites in February, mussels from St. Brieuc had concentrations of chromium (Cr), molybdenum (Mo), nickel (Ni) and titanium (Ti) overall 2-4x higher than those of Brest and Lannion. The Lannion site was distinguished by iron (Fe) and aluminum (Al) concentrations that were more than 2x lower than the other two sites. In contrast, lead (Pb) concentration was highest in Brest, more than 5x higher than that of the mussels from Lannion and St. Brieuc, and exceeding the ML. Regarding temporal variation of chemical compounds in Brest mussels, Pb concentration was similar in February and April with a twofold decrease in September; Fe and Al concentrations showed noticeable decrease over time. The highest concentrations were found in winter and a general decrease was observed throughout the study period.

Table 2. Trace element concentrations (mg/kg w.w.) in mussel tissues collected from three bays in northern Brittany: Brest (BRE), Lannion (LAN) and St. Brieuc (STB), in February 2017, and in Brest in April and September 2017. Limit of detection: < 0.002 mg/kg). *ML: Maximum Level set by the Commission Regulation (EC) No. 1881/2006 amended by (EC) No. 629/2008 and (EC) No. 420/2011.*

	February			***April***	***September***
Chemical compounds	**STB**	**LAN**	**BRE**	**BRE**	**BRE**
Cd (ML: 1.0)	0.07	0.07	0.13	0.14	0.10
Hg (ML: 0.5)	0.01	0.02	0.04	0.03	0.02
Pb (ML: 1.5)	0.32	0.23	1.62	1.54	0.70
Co	0.15	0.09	0.21	0.12	0.07
Cu	1.42	1.35	1.43	1.36	1.59
Cr	3.80	1.49	0.99	0.54	0.09
Fe	175.98	62.28	157.48	85.65	34.10
Mn	3.15	1.79	2.69	1.81	1.22
Mo	0.84	0.37	0.23	1.32	0.14
Ni	2.31	1.00	0.65	0.49	0.12
Zn	13.7	15.84	15.85	18.73	12.32
Ti	5.90	1.57	1.91	0.95	0.38
Sn	<0.02	<0.02	<0.02	<0.02	0.19
Al	173.11	65.85	143.72	69.09	22.70
Se	1.28	1.48	1.39	1.04	0.85
As	3.16	3.99	3.15	6.42	3.41

4. Discussion

An *in situ* study combining various analytical procedures was performed to explore causes of mussel mass mortality. The experimental design for field work involved assuming the culture procedures and using the mussel batches of the professional mussel farmers, taking advantage of their activity to avoid any element or circumstance different from the common

311 practice that could distort the usual mussel performance through on-growing. This approach
312 provided an accurate representation of what was occurring on the farms and was chosen as a
313 first exploration assuming that more refined experimental designs eventually may be needed
314 while taking advantage of the results of this first approach.
315
316 ### *4.1. Temperature, conditioning and mortality*
317 *Mytilus edulis* is known to resist extreme temperatures (Aarset, 1982; Seed &
318 Suchanek, 1992; Almada-Villela *et al*., 1982), however, the thermal profiles observed in our
319 sites were not extreme or unusual. The narrower temperature range observed in Lannion was
320 probably due to the farming method because mussels on subsurface longlines are always
321 submerged and undergo less marked temperature variation than mussels on intertidal stakes.
322 Results showed that a 'mortality window' opened in all sites during the spring season; similar
323 observations also have been reported recently in France by other researchers (Benabdelmouna
324 *et al*., 2018; Dégremont *et al*., 2019). This period corresponded to the spawning season of
325 mussels. Gonad development usually begins in October/November, and by the end of the
326 winter the gonads are ripe (Gosling, 2003). All energy accumulated during the quiescent
327 period is used to fuel gametogenesis and finally spawning; thus, when mussels spawn, they
328 are in poor condition during the remainder of the spring period, with low glycogen content
329 (Najdek & Sapunar, 1987). Mussel growth is primarily influenced by the reproductive stage
330 and food availability (Gosling, 1992) but growth capacities also have been linked to site and
331 animal origin (Dickie *et al.*, 1984). The higher C.I. of mussels from Lannion in May could be
332 explained by (i) their better growth capacities due to their origin, (ii) the greater trophic
333 richness of the site, and (iii) the subtidal farming –which allows them continuous access to
334 trophic resources. Indeed, Prou and Goulletquer (2002) showed that mussels grown on
335 longlines had higher growth performance than mussels grown on *bouchot*. However, this did
336 not prevent mortality. Primary production, and thus food availability, are reduced during

winter periods when seawater temperatures are below 10°C (Cloern, 1996) and mussels undergo long-time starvation periods (Harbach & Palm, 2018). *Mytilus* sp. can handle longer food restriction periods and maintain its shell size by using energy from its own tissue (Dare & Edwards, 1975; Riisgård *et al*., 2014). However, no link between C.I. of mussels and mortalities obtained in site was found.

These various data and observations clearly illustrate that this transition period between winter and spring –corresponding to the spawning period– is stressful for mussels and decisive in their survival. Thus, spawning stress could contribute to mussel weakening and to an increased mortality rate to some extent. However, considering the very similar C.I. observed between the different sites studied –between those with high mortality and those with low mortality– this parameter alone cannot explain abnormal mortality.

4. 2 Mussel symbionts

Villalba *et al.* (1997) classified mussel symbionts in three groups according to their pathogenicity. Among those identified in this study, ciliates are in the first group with unnoticeable pathogenic effects, *Mytilicola* sp. are in the second group that may damage the host, but are not lethal, and *Marteilia* sp. and trematodes are in the third group with potentially lethal effects.

Figueras *et al.* (1991) observed that, even at the highest densities of infestation, ciliates caused no detectable tissue damage in gills or in digestive tubules. More recent detailed studies of the intracellular ciliates in digestive tubules reported similar conclusions (Fichi *et al*. 2018). Our results are consistent with those reports, no associated inflammation or abnormal mortality was detected in St. Brieuc, the site most affected by ciliates. Regarding *Mytilicola* sp., Brienne (1964) and Blateau (1989) observed weight loss in French mussels infested with this copepod at the end of winter and early spring (breeding period); the mortality it caused was related to the decrease in mollusc vitality. Nevertheless, in most cases,

despite a high infestation intensity reported in English and Spanish mussels, it is evident that the host population can sustain the infestation indefinitely (Davey, 1989; Robledo *et al.*, 1994). Again, our results were consistent because the highest prevalence of this copepod was recorded in St. Brieuc and no abnormal mortalities were observed over the period at this site; the mortality peak observed in May could be attributed to weakness due to the breeding season, which could be intensified by the presence of *Mytilicola* sp. as observed by Brienne (1964) and Blateau (1989).

Lauckner (1983) found that digenetic Trematoda were the most frequent and important metazoan parasites of bivalves. Indeed, trematode sporocysts and metacercaria were capable of causing a wide range of harmful consequences in their hosts (lesions, compression of tissues, castration, and deep weakness) that can lead to death when infestation is heavy (Robledo *et al.*, 1994; Laruelle *et al.*, 2002). Bakhmet *et al.* (2017) found a metabolic level supported by significant growth deficiency in parasitized *M. edulis*. This also was observed in Normandy (France) by Le Breton & Lubet (1992), as well as a variation in parasitism over time as we observed in Brest, and an increase in mortality between January and July.

No significant statistical link was established for *Marteilia* sp., but the p-value close to 0.05 should be highlighted as it was entirely influenced by results from Brest. The presence of *Marteilia* sp. only in the most affected site should not be neglected, especially because it not only severely harms flat oysters, *Ostrea edulis*, but also *M. edulis* and *M. galloprovincialis* (Villalba *et al.* 1993; Villalba *et al.* 1997; Fuentes *et al*., 1995; Arzul *et al*., 2014). Indeed, several studies have shown that high mortality rates observed in certain mussel populations in Spain and France were positively correlated with the presence of this parasite (Villalba *et al*., 1993; Garcia *et al.*, 2005). Marteiliosis leads to the arrest of growth due to the degradation of digestive cells, loss of glycogen in tissues and resulting in considerable weight loss of the mollusc (Grizel & Tigé, 1973). This disease could explain the lower C.I. in Brest. Marteiliosis also causes a general weakening of the host, promoting opportunistic parasite

development (bacteria, ciliates) that could cause secondary infestations (Grizel, 1985). For Brest mussels, no significant changes were observed between the C.I. for weight calculated in February and those calculated in September. This is not typical because mussels should be fleshy at the end of summer and require accumulated energy reserves for gonadal development and winter survival. Previous studies in flat oysters showed that mortality due to *Marteilia* sp. was observed just after the breeding period during which the animal consumes a lot of energy. Marteiliosis also is known to cause hemocytic infiltration and occasionally granulocytomas, which cause the destruction of the pathogen but also of the host tissues (Villalba *et al*., 1993). These effects were observed in Brest. The drastic decrease in prevalence observed in Brest between May and September could be explained by death of infected mussels. Histological examination does not allow discriminating between the congeneric species *Marteilia refringens* and *M. pararefringens*; although both species have been detected in mussels, *M. pararefringens* is more frequently reported (Kerr *et al*. 2018). The infected samples analysed with the real time PCR procedure showed infection with *Marteilia* sp.

Each of the symbionts and parasites detected in the study have a varying degree of negative effect on their hosts. A single non-lethal parasite may cause some damage but not death, but the cumulative effect of different non-lethal parasites may have an impact on the survival of the host. This is particularly true during a harsh season and at a key stage in the life cycle of the mussel. It is clear from our data that *Marteilia* was one of the major factors contributing to mortalities in Brest.

4.3 Lesions and alteration: hemocytic infiltration, granulocytomas and neoplasia

Some cases of hemocytic infiltration (positively correlated with mortality) and granulocytomas were not linked to a specific pathogen. It should be noted that abnormal is the most frequent alteration and this is considered to be an important biomarker of lesion and

inflammation in bivalves (Cuevas *et al.,* 2015). Cuevas *et al*. (2015) showed that mussels from the most impacted sites (by metallurgic and shipyard activities) endured the most significant deleterious effects showing inflammation. Also, Sheir & Handy (2010) established a potential link between hemocytic infiltration and lesions with the presence of xenobiotics. Lowe & Moore (1979) stated that granulocytomas reflect symptoms of long-term exposure to contaminants; the same observation was made about a potential correlation between neoplasia and water pollution because neoplastic disorders have been reported in bivalves collected from polluted areas (Lauckner, 1983; ICES, 2017).

In our study, pollution alone cannot explain the presence of granulocytomas. In May, they were more frequent in Lannion, the site with the lowest concentrations of chemical compounds. In addition, the highest prevalence of granulocytomas in Brest were observed in April and September while in these two months, the records of trace elements showed lower concentrations, more specially for lead. Finally, the prevalence of granulocytomas recorded in St. Brieuc was close to that of the other two sites and hemocytic infiltration and neoplasia were not recorded, while the concentration of all trace elements, except for lead, was higher in this site than in the others. Villalba *et al.* (2001) reported association of cockle, *Cerastoderma edule,* mortality with lesions resulting from heavy inflammatory reaction without a clearly identified cause, and with disseminated neoplasia. We observed inflammations with heavy hemocytic infiltrations correlated with mortality in Brest and Lannion, but not always associated with a specific cause. Disseminated neoplasia is a progressive disease insofar as neoplastic cells proliferate and replace normal hemocytes in circulation and could lead to death (Elston *et al.,* 1988; Ciocan & Sunila, 2005). Because hemocytes are cells with a key role in many physiological functions (digestion, excretion, nutrition, defense mechanisms; Cheng, 1984; Fisher, 1986, 1988), heavily affected mussels have reduced abilities and usually die (ICES, 2017). Indeed, mussels affected by disseminated neoplasia had, after the decrease in the number of normal circulating hemocytes, a weakened defense system and showed

reduced bacterial clearance (Kent *et al.*, 1989). Mass mortalities of various bivalve species have been associated with disseminated neoplasia (see reviews by Elston *et al*., 1992; Carballal *et al.*, 2015). In France, Benabdelmouna & Ledu (2016) observed that genomic abnormalities, namely aneuploidy of the hemolymph cells, were significantly correlated with *Mytilus* sp. mortalities observed in 2015 in the French Atlantic coast. Furthermore, Benabdelmouna *et al.* (2018) linked the occurrence of a significant percentage of aneuploid hemolymph cells to disseminated neoplasia and concluded that this disease could be viewed as a major cause of morbidity and mortality for French mussels. Elston *et al.* (1988) followed the evolution of this disease over 4 months in *M. trossulus*; they reported a relatively long dynamic progression of the disease in the early stages (2-3 months) and then a quick evolution when disseminated neoplasia leads to rapid death. This is consistent with the mortality dynamics observed in our study and the absence, or possibly undetected early-stage cases of disseminated neoplasia in February. A relatively high prevalence of 18% was observed in Brest in April and therefore most certainly contributed to a percentage of mortality during this period and/or may also have been an aggravating factor with an already present pathology; but abnormal mortalities began in February with no sign of disseminated neoplasia being observed at that time. Lannion presents a similar pattern; in May few cases were found with a prevalence of 2.4% but no signs in February. We hypothesize that if histological observations on Lannion mussels had been made in April, a higher rate of neoplastic mussels would have been observed and may have played a role in the observed mortality percentage.

We showed that inflammation and hemocytic infiltration were correlated with mortality, but it was not always possible to link them to an identified cause. In Brest, pollution could have a role (see below) but for Lannion, the cause(s) remain unknown.

4.4 Chemical contamination

First, and in relation to what has been previously discussed, when food availability is low and competition between individuals is high, mussels increase their levels of water uptake through their gills. This leads to congestion and accumulation of useless substances in their tissues, including pollutants, which could result in increase of mortality rates (Dailianis, 2010). It is known that anthropogenic compounds and heavy metals play roles in defence capacities of bivalve molluscs and could increase susceptibility to disease (Coles *et al.*, 1995; Pipe & Coles, 1995; Morley, 2010). In addition, the effects of environmental contaminants sometimes correspond to a direct toxic action on tissues or cells (Gagnaire *et al.*, 2004).

Some of the elements analyzed, Hg, As, Cd, Pb and Sn, have no known biological function and, except for Sn, are included in the list of the ten chemicals of major public health concern as part of International Programme on Chemical Safety (World Health Organisation, 2010). These elements are natural trace components of the aquatic environment, but their levels increase due to agricultural, industrial and mining activities. Even low metal concentrations may threaten the health of aquatic and terrestrial organisms, humans included (Sarmiento *et al*., 2011). Among all the metallic elements that Moschino *et al.*, (2016) have measured in mussel tissues (As, Cd, Cr, Pb, Al, Fe, Hg, Cu, Ni, Zn) during an *in situ* study over several years, a positive correlation between mussel mortality rate and Pb, Fe and Al was observed. Furthermore, the average concentrations they measured in the mussel tissues for these three elements were comparable with ours. It should also be noted that for Fe and Al, relatively similar high concentrations were found in St. Brieuc and Brest, while for lead, only mussels from Brest were above the threshold. Several biomarkers representative of the health status of the aquatic environment have been identified in mussels (Depledge, 1994; Dailianis, 2010), and it was shown that mussels have lower defense mechanisms against metal oxidative challenge and toxicity than oysters. Indeed, Funes *et al*. (2006) have shown that activities of antioxidant enzymes are insufficient (compared to those observed in the Pacific oyster, *C. gigas*), which means that mussels are not sufficiently protected from the oxidative stress

associated with metal pollution. In addition, Viarengo *et al.* (1991) and Petrović *et al.*, (2004) observed seasonal variations in antioxidant responses related to physiological processes and showed inhibition of these defense mechanisms in winter related to the reproductive period and gonad resorption.

From the Chemical Contamination Observation Network of Ifremer (ROCCH), the Bay of Brest is known to be one of the sites most affected by lead contamination on the French coast for many decades (the first most contaminated on the west side of France) (Belin *et al*., 2013). According to these authors and local authorities (*com. pers*.), the high Pb concentrations found in Brest mussels are mainly explained by the presence of silver lead mines around the Aulne river (surface area: 1842 km²) several kilometers upstream (at Poullaouën and Huelgoat, Finistère, France). The river provides more than 63% of the bay's freshwater supply (Auffret, 1983).

Lacroix *et al.* (2015) observed differences in physiological responses to contaminants between native Brest Bay and imported mussels. Non-natives were more sensitive and showed more sensitive biomarker responses. Therefore, it can be supposed that mussels caught in a more open and less contaminated marine area will be strongly impacted and stressed when they arrive in a more polluted area. Lacroix *et al.* (2017) also observed an altered physiological state, early spawning, in mussels in a polluted area (Bay of Brest) compared to those on the Atlantic coast of Brittany (under oceanic influence) suggesting that their health is compromised at this period. Therefore, the pollution in Brest is an additional deleterious factor to others previously discussed and could be an explanation for the heavy hemocytic infiltrations not found to be linked to a specific pathogen. However, this is probably not the case for Lannion, an unpolluted site.

4.5 Bacterial profiles

Species belonging to the genus *Pseudoalteromonas* are widely distributed in marine environments globally. Some studies have shown a positive role of *Pseudoalteromonas* sp. biofilms in the settlement of mussel, *Mytilus coruscus* larvae (Yang *et al.*, 2013; Li *et al*., 2014). On the other hand, Venkateswaran & Dohmoto (2000) identified a species of *Pseudoalteromonas* which play a natural antifouling role due to the products it excretes, which prevent fixation of mussel byssal threads. However, no pathogenic or lethal role has been demonstrated by this species on bivalves. Species of the genus *Pseudoalteromonas* and *Vibrio* are part of the normal microflora of temperate water marine animals and their proportion increases during the spoilage of the bivalves (Gram & Huss, 1996; Madigan *et al*., 2014). Also, Lokmer & Wegner (2015) showed that host-associated microbial communities are linked to abiotic and biotic factors.

The genus *Vibrio* includes a ubiquitous, diverse and abundant temperate coastal marine bacterial community (Thompson *et al*., 2004). The species of the genus *Vibrio* show a very high diversity; more than 110 species have been identified, each with different relationships with their hosts, ranging from symbiosis to significant pathogenicity (Travers *et al.*, 2015). Among those that appear to be pathogenic to bivalves, species belonging to the Splendidus clade are systematically highlighted. For example, Béchemin *et al.* (2015) identified *V. splendidus*-related species in moribund mussels during mussel mass mortality outbreaks in summer 2014 in France; these isolates appeared to be capable of inducing mortality under laboratory conditions. Also, Ben Cheikh *et al.* (2016, 2017) observed that a pathogenic strain of *Vibrio splendidus* clade inhibits the immune response in *M. edulis* by altering hemocyte function and viability and causes hemocytic infiltration and granulocytomas. The high genotype diversity of the bacteria belonging to the Splendidus clade and the dynamic nature of microbial communities complicates considerably efforts to elucidate the role of *V. splendidus* clade bacteria in vibriosis (Kwan & Bolch, 2015). In addition, *Vibrio* studies are culture dependent and specialists themselves admit that they do

not know if the dynamics they observed in bacteria populations reflect physiological changes in a viable but non-cultivable state or fluctuations in density with environmental parameters (Thompson *et al*., 2004). Knowledge of their role as animal pathogens and their mechanisms of action in pathogenesis has been limited. Indeed, Bruto *et al*. (2018) recently showed that, within the Splendidus clade, virulence represents an ancestral trait, but it has been lost from several populations. They identified two *loci* necessary for virulence and can now associate virulence in bivalves with one or many specific *V. splendidus* strains. Thus, simply finding some *V. splendidus* strains in mussels is no longer sufficient to incriminate them during a mortality episode. Accordingly, since *V. splendidus* was found in all sites, no matter the mortality rate, assessing whether these two virulence genes are present or not in our isolates would be highly interesting.

To explain abnormal mortalities, to identify a 'biotic disease' with one identifiable lethal pathogen (virus, bacteria or parasite) is desired, but there is evidence that surrounding conditions (abiotic factors like pollution, temperature and seasons), as well as the presence of multiple pathogens and parasites affecting the condition of animals are inseparable and cannot be considered individually. Because many factors could be involved, it is complicated to understand precisely mortality factors that have very high inter-site and inter-annual variability. Some factors can lead to gradual changes that progressively disrupt animal homeostasis while others can cause acute mortality. Dare (1976) concluded that mortality in mussel populations resulted from an interaction between several physical and biological factors. Similarly, Lokmer & Wegener (2015) argued that, in addition to the presence of pathogens, environmental factors have a strong impact on disease efficacy and mortality. In addition, it seems that genetics could also have an impact on mortality (Dickie *et al*., 1984; Mallet *et al.*, 1987; Dégremont *et al*., 2019).

Our experimental design, using different mussel stocks corresponding to different geographic sources in the different experimental sites, did not allow evaluating the influence

of the genetic background on the mussel mortality, that is to say if mussel stocks from different geographic sources would show different susceptibility to mortality outbreaks. Mussels on the French Atlantic coast have complex genetic structure (Bierne *et al.*, 2003; Fly *et al*., 2015; Michalek *et al.*, 2016, Simon *et al*, 2019).

Obviously, the possibility of missing an unknown and yet undetectable or uncultivable pathogen should not be excluded. Furthermore, a complex 'pathosystem' between two or more pathogens, as shown by De Lorgeril *et al.* (2018), may also exist and would require an integrative and holistic approach to be understood as recommended by these authors in the case of multi-factorial diseases.

5. Conclusion

This multi-parametric field study, carried out on commercially cultivated mussels, was a first approach to identifying potential causes of mortality in northern Brittany. We completed a first sorting and identified important factors for future experimental studies. In Brest, the presence of *Marteilia*, inflammatory lesions and pollution, with additional weakening factors such as the breeding period, explains part of the mortalities. In Lannion, no relevant parasites or pollution were found, although heavy inflammatory lesions were observed; thus, further research is required to explain those hemocytic infiltrations and to determine the cause(s) of the mortality observed in this site.

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Additional file 1: Supplementary Materials and Methods

PCR screening for bivalve mollusc known pathogens

For each mussel, a pool of minced tissues from foot, adductor muscle, gills, mantle, and digestive gland was subjected to DNA extraction using a QIAamp DNA minikit® (Qiagen, Courtaboeuf, France) following the manufacturer's protocol for blood or body fluids, except for elution performed in 60 µL Qiagen elution buffer AE. The quality of the extracted DNA was checked with NanoDrop™ 2000c spectrophotometer (ThermoFisher Scientific™, Waltham, MA USA). Within the same batch, the individual extracts were pooled in groups of 5 to have 4 replicates per batch for each PCR done. The known bivalve pathogens investigated by TaqMan® real-time PCR and classical PCR were: *Bonamia* spp., *Haplosporidium nelsoni*, *Marteilia* sp., *Mikrocytos mackini*, *Nocardia crassostreae*, Ostreid Herpesvirus type 1 (OsHV-1), *V. aestuarianus*, *V. tubiashii*, *V. harveyi*, and *V. splendidus* (the true species *V. splendidus* of the Splendidus clade named here Splendidus cluster) (Table S1). Amplification reactions were performed in a total volume of 25 µL using a SmartCycler® (Cepheid, USA). Each reaction contained 12,5 µL of a Premix Ex Taq® 2 X Takara®(Lonza, Verviers, Belgium), 9 µL of purified water, 2 µL of DNA sample (replaced with 2 µL of purified water in the negative control) and 0.5 µL of each primer (20 µM) and probe (10 µM) or SYBR Green (for *N. crassostreae*). Each assay included negative and positive control reactions. The thermal cycle profile consisted of 95 °C for 10 s followed by 40 cycles of 95 °C for 5 s and 60° for 30 sec (or 62 °C for *V. splendidus* or 64 °C for *V. harveyi*). Beforehand, for each real-time PCR developed for this study, the inclusivity and exclusivity were tested. Moreover, in order to exclude false-positive results, when a signal was obtained, the amplicon was sent to Eurofins MWG Operon (Ebersberg, Germany) to be purified and subsequently sequenced; only then, after using the NCBI BLAST, confirmation of the targeted pathogen could be made.

Table S1. Target genes, primers (forward (Fw) and reverse (Rv)) and probe (Pr) used for detection of known bivalve molluscs pathogens.

Name of pathogens	**Target gene**	**Oligo-nucleotides sequences (5'-3')**	**References**
Bonamia spp.	28S rRNA	Fw TCCCTGCCCTTTGTACACA Rv CTCTTATCCACCTAATTCACCTCAG Pr TxR-CGCCCGTCGCTTCTACCGATT-BHQ2	Present paper
Haplosporidium nelsoni	SrRNA	Fw CACGCGCGCTACAATGT Rv CGAGATTACCCGGCCTTCT Pr FAM-CACGCAACGAGTTCAACCTTGCC-BHQ1	Present paper
Marteilia sp.	ITS1	Fw CACACTACTCTTCGCTTTCGAT Rv GACTACCCGTGCCGAACA Pr Cy3-TCGCAAACAGGAAGCGGCTCTC-BHQ1	Present paper
Mikrocytos mackini	28S rRNA	Fw GGTGGCCGAATGACGTAGT Rv GCCTATGACAGCACGAAGCA Pr Cy5-CCGCTTCGGCGTGCAGTCTC-BHQ2	Present paper
Nocardia crassostreae	16S-23S rNA ITS	Fw CCTCGATACCGCCGAAGAA Rv CAACACACCCGCATCAAA	Carrasco *et al.* (2013)
OsHV-1	B region	Fw GTCGCATCTTTGGATTTAACAA Rv ACTGGGATCCGACTGACAAC Pr TxR-TGCCCCTGTCATCTTGAGGTATAGACAATC-BHQ2	Martenot *et al.* (2010)
V. aestuarianus	dnaJ	Fw GTATGAAATTTTAACTGACCCACAA Rv CAATTTCTTTCGAACAACCAC Pr TxR-TGGTAGCGCAGACTTCGGCGAC-BHQ2	Saulnier *et al.* (2009)
V. tubiashii	vtpA	Fw GGTACGGACTATCCGGGATT Rv TTCACCGCTGAGTTGTTCAT Pr Cy3-ATCGTCGATAAATCAGGCACAACCTGT-BHQ1	Present paper
	vth	Fw CGGTTGATATTCGCGTCAA Rv GTGTGAAACCCTGCGAAGTA Pr Cy5-TATCACAGATGCGCTCGGTTCAGTC-BHQ2	Present paper
V. harveyi	16S rRNA	Fw CGAGCGGAAACGAGTTATCTG Rv CTCACCAACTAGCTAATCCCACCTA Pr TxR-CCGCATAATACCTACGGGTCAAAGAGGG-BHQ2	Present paper
V. splendidus (Splendidus cluster)	toxR	Fw AGCAGCGGCTGAAATTGCA Rv GGCCGCAGTTGGTGTTGTT Pr FAM-CAATGACTGAAGCTGTCGAGCCC-BHQ1	Oden *et al.* (2018)