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

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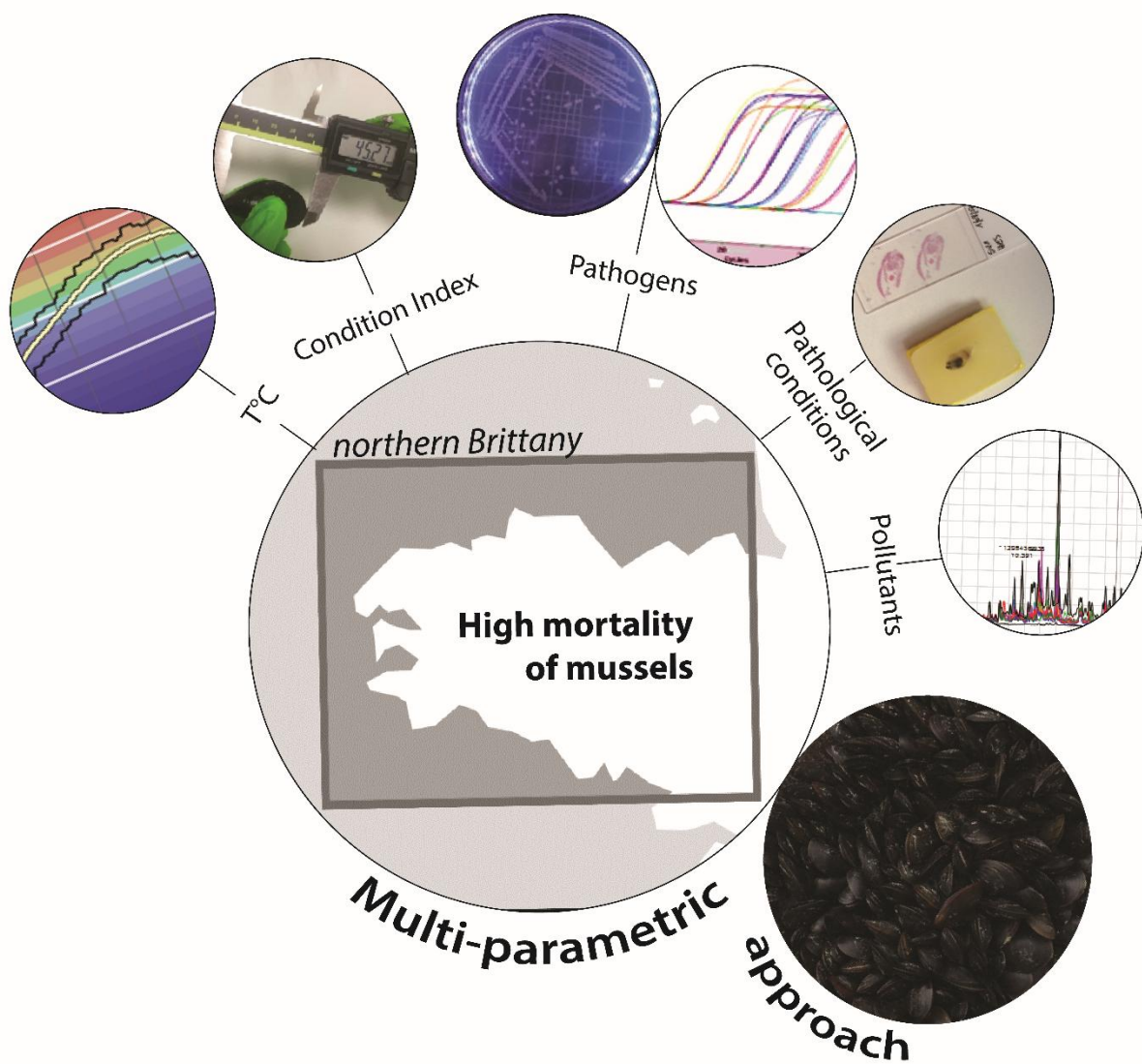
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20 Highlights

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

27

28 Graphical abstract



29

30

31 **Abstract**

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

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

47

48

49 **1. Introduction**

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

61 In 2014, France was affected by high and unusual mass mortality of mussels, both
62 juveniles and adults, affecting several important production areas along the French coast of
63 the Atlantic and English Channel. Production losses reached 50 - 100% depending on the sites
64 (Béchemin *et al.*, 2015; François *et al.*, 2015; Allain & Bernard, 2016; Lupo *et al.*, 2016). The
65 phenomenon again affected farms on the west coast of France in the first quarter of 2016
66 (FAO, 2016). Unlike mortality events in Pacific oyster (*Crassostrea gigas*), very few massive
67 mortalities of *Mytilus* spp. occurred along the French coast until the winter of 2014 (Brienne,
68 1964; Bateau, 1989; Guichard *et al.*, 2011).

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

75 Brest, located at the extremity of the Brittany Peninsula, is one of the locations where mussels
76 suffered massive mortality in 2014 and beyond. Estimated losses were 30% to 80%,
77 depending on the year. *Vibrio* strains and pathogenic bacteria belonging to the Splendidus
78 clade were identified and associated with these mortalities (François *et al.*, 2015; Lupo *et al.*,
79 2016). Other researchers have suggested that the cause of mussel mortalities is disseminated
80 neoplasia. This disease is well documented in marine bivalves all over the world (Peters,
81 1988; Elston *et al.*, 1992; Landsberg, 1996; Carballal *et al.*, 2015) and, in some bivalve
82 species including the mussel *Mytilus trossulus*, spreads by transmission of cancerous cells
83 between individuals (Metzger *et al.*, 2015, 2016). Some scientists observed chromosomal
84 abnormalities associated with neoplasia in *Mytilus trossulus* (González-Tizón *et al.*, 2000)
85 while advanced neoplastic disease was associated with significant mortalities of *Mytilus* sp.
86 (Moore *et al.*, 1991), including *M. trossulus* in Washington and Oregon (USA) and in British
87 Columbia (Canada) (Elston *et al.*, 1992). In France, Benabdelmouna & Ledu (2016) and
88 Benabdelmouna *et al.* (2018) presented evidence of the involvement of genomic
89 abnormalities in mortality outbreaks in blue mussels and linked them to disseminated
90 neoplasia. In addition, some cases of disseminated neoplasia in *Mytilus edulis* from Lannion
91 (northern Brittany, France) were observed in mid-October 2016 by Burioli *et al.* (2017)
92 during a mortality event.

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

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

109

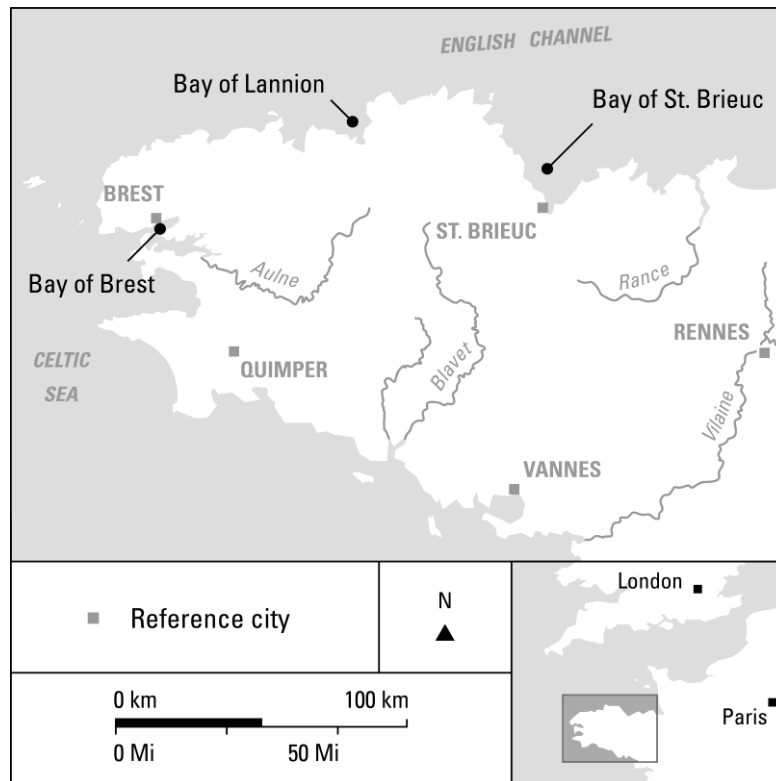
110 **2. Materials and methods**

111

112 ***2.1. Sampling sites and mussels***

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

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



129
130 Figure 1: Location of the three sampling sites: the bays of Brest, Lannion and St. Brieuc.
131

132 **2.2. Mortality and temperature monitoring**

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



143

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

147

148 ***2.3. Sampling for analysis***

149 Sampling for analysis was carried out with different frequency among sites: February, April,

150 May and September 2017 for Brest, and February and May 2017 for Lannion and St. Briec

151 (Table 1). At each sampling, 50 mussels per site were collected from baskets for histology,

152 and 20 additional mussels for biometry, molecular biology, bacteriological and chemical

153 analyses (performed on the same animals). The collected mussels were transported in thermal

154 bags to the laboratory and either analysed immediately or held in a refrigerator (4 °C)

155 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	<i>Bouchot</i>	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. Briec	48°33'07.6"N	2°38'55.6"W	<i>Bouchot</i>	27/02/2017		28/05/2017		50 per date (total=100)	50 per date (total=100)	20 per date (total=40)

1 **2.4. Biometry**

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

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

5

6 **2.5. Histological examination**

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

18

19 **2.6. Bacteriological analysis**

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

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

35

36 **2.7. Molecular analyses**

37 The first step to determine the bacterial profile of the mussel batches consisted in
38 discriminating between strains related to the *V. splendidus* clade (*V. splendidus*-related
39 species) and other marine bacteria, as some vibrio strains belonging to this Splendidus clade
40 are known to be pathogenic for molluscs. One TaqMan[®] real-time PCR, targeting the *16S*
41 *rRNA* gene of *V. splendidus*-related strains (PCR1) (Oden *et al.*, 2016), was carried out on a
42 Smart Cycler[®] (Cepheid, USA); the primers used for PCR1 were *SpFI*
43 5'ATCATGGCTCAGATTGAACG3' and *SpRI* 5'CAATGGTTATCCCCACATC3' (Nasfi
44 *et al.*, 2015) and the probe *SpProbe* 5'CCCATTAACGCACCCGAAGGATTG3'. The
45 reaction volume of 25 μL contained 12.5 μL of Premix Ex Taq[®] 2 X Takara[®] (Lonza,
46 Verviers, Belgium), 0.5 μL of each primer (20 μM), 0.5 μL of probe (10 μM), 9 μL of purified
47 water and 2 μL of extracted DNA (replaced with 2 μL of purified water in the negative
48 control). The thermal cycling profile was 95°C for 10 s, followed by 40 cycles at 95 °C for 5 s
49 and 62 °C for 30 s. When PCR1 was positive, a conventional PCR targeting the housekeeping
50 genes *mreB* of *V. splendidus*-related strains (PCR2) was performed because it is the most
51 discriminant gene for the identification of closely related strains among the Splendidus clade

52 (Oden *et al.*, 2016). The primer pair for PCR2 was *mreB*-F
53 5'CTGGTGCTCGYGAGGTTTAC3' and *mreB*-R
54 5'CCRTTYTCTGAKATATCAGAAGC3'. For colonies not identified as members of the
55 Splendidus clade (PCR1 negative), another conventional PCR targeting the *16S rRNA* gene
56 (PCR3) (Burioli *et al.*, 2018) was done; the primer pair for PCR3 was *16S27*-F
57 5'AGAGTTTGATCMTGGCTCAG3' and *16S1492*-R 5'ACCTTGTTACGACTTCAC3'. For
58 the conventional PCR, typical 25- μ L reaction mixtures contained 12.5 μ L of Premix Ex Taq[®]
59 2 X Takara[®] (Lonza, Verviers, Belgium), 0.5 μ L of each primer (20 μ M), 9.5 μ L of purified
60 water and 2 μ L of DNA template (replaced with 2 μ L of purified water in the negative
61 control). Conventional PCR amplifications were carried out in a T100[™] Thermal Cycler
62 (Bio-Rad, France) and the thermal program was as follows: 10 s at 95°C; 30 cycles for 10 s at
63 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
64 then analysed with QIAxcel[®] Advanced System (Qiagen, Courtaboeuf, France) and those
65 with the expected size were sent to Eurofins MWG Operon (Ebersberg, Germany) to be
66 purified and sequenced. Species were identified using the National Center for Biotechnology
67 Information (NCBI) Basic Local Alignment Search Tool (BLAST) with individual *16S*
68 *rRNA* sequences, and *mreB* sequences were aligned using a multiple sequence alignment
69 Multiple Sequence Comparison by Log-Expectation (MUSCLE). Phylogenetic analyses were
70 performed in MEGA7 (Kumar *et al.*, 2016) using the Neighbor Joining method (Tamura *et*
71 *al.*, 2013) and the maximum composite likelihood model with a bootstrap of 1000 replications;
72 sequences of the *mreB* gene from 44 different reference bacterial strains from the Splendidus
73 clade were used (see 2.1. in Oden *et al.*, 2016). *V. aestuarianus* 02/041, *V. ordalii* 12B09 and
74 *V. penaeicida* AQ115, were provided from the Genomic of *Vibrio* Research Department
75 (CNRS Roscoff, France) and used as the Splendidus clade outgroup.
76 In parallel, the presence of known bivalve pathogens (*Bonamia* spp., *Haplosporidium nelsoni*,
77 *Marteilia* sp., *Mikrocytos mackini*, *Nocardia crassostreae*, Ostreid Herpesvirus type 1

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

80

81 **2.8. Chemical analyses of flesh**

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

87 Presence of trace metal compounds (Pb, Hg, Cd, Co, Mo, Sn, Cr, Cu, Ni, Zn, Fe, Al, Mn and
88 Ti) and non-metallic trace elements (As and Se) in mussel flesh was quantitatively determined
89 by inductively coupled plasma-mass spectrometry (ICP-MS Triple Quad 8800, Agilent
90 Technologies, Santa Clara, CA, USA) after wet digestion through high-quality grade mineral
91 acids (HNO₃, Analpure[®], Analytika[®], Prague, Czech Republic) and oxidants (H₂O₂,
92 Normapur[®], VWR international, Radnor, Pennsylvania, USA), following previously described
93 protocols (Squadrone *et al.*, 2016). All analyses were calibrated against analytical standards.
94 The limit of quantitation was 0.02 mg/kg for elements analysed by ICP-MS. All
95 concentrations are given on a wet weight (w. w.) basis.

96

97 **2.9. Data analysis**

98 **2.9.1. Half-stock index**

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

104 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
105 which describes these two points:

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

106 To obtain a more tangible index, the time (in days) needed to halve the population was used:
107 $d_{0.5} = \ln(2) / \lambda$. Therefore, this index corresponds to the number of days it takes to halve the
108 population with the measured mortality rate, in a very similar way to half-life of radioactive
109 elements. The lower this index, the higher the mortality rate and decrease of the stock. In the
110 absence of mortality, this index tends towards infinity. For a better graphical representation, a
111 maximum value was assigned to this index equal to 1500 (which corresponds to a loss of 15%
112 over 1 year).

113 2.9.2 Statistical analysis

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

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

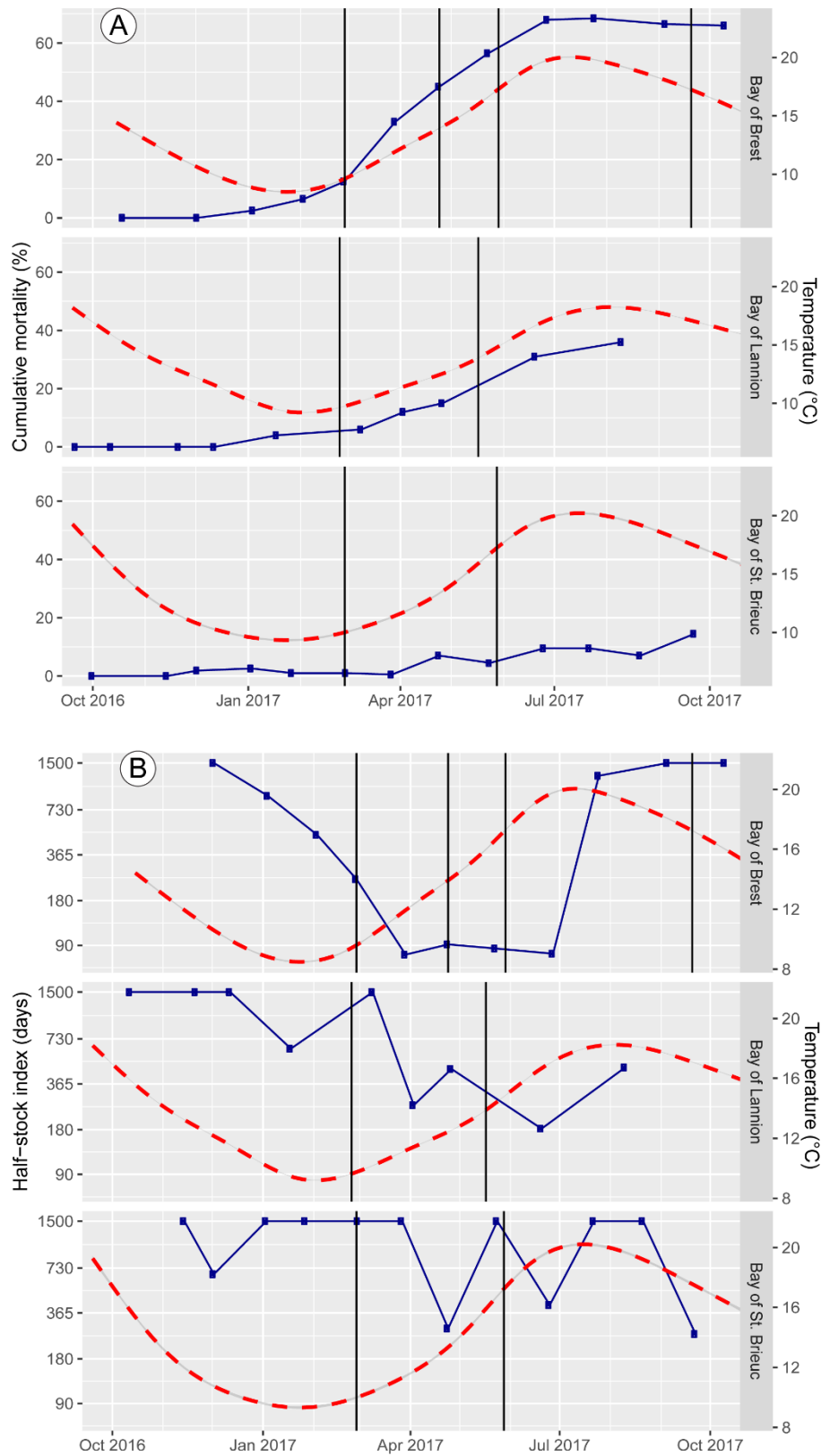
129 **3. Results**

130 **3.1. Mussel species, temperature monitoring and mortality**

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

133 Regarding thermal profiles (Fig. 3), Lannion appears to be a rather temperate site compared to
134 those of Brest and St. Brieuc, which showed wider temperature variation, especially higher
135 temperatures in summer. From February to August, the temperature of Lannion rose from 9 to
136 18 °C, that of St. Brieuc from 9 to 20 °C and that of Brest from 8.5 to 20 °C.

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



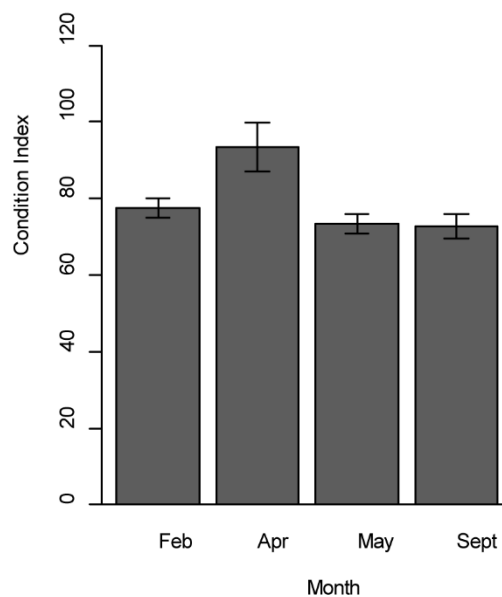
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149 Figure 3: Variation of temperature (broken line) and: (A) cumulative mortality (%) observed over the duration of
 150 the study based on monthly mortality monitoring (continuous line with dots); (B) half-stock indices (days)
 151 calculated monthly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

153

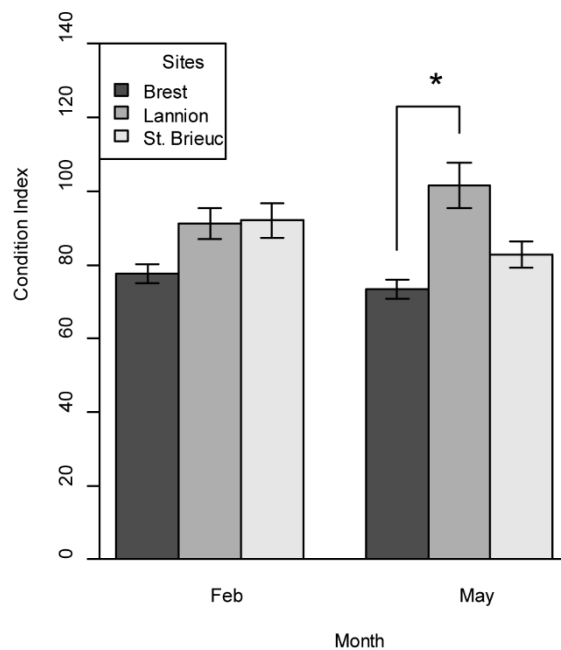
154 **3.2. Biometry**

155 When the C.I. of Brest mussels at the four sampling dates between February and September
156 2017 were compared, despite an upward trend in the April index, no significant difference
157 was observed (ANOVA, $p > 0.05$) (Fig. 4). When the C.I. of mussels from the three sites on
158 both dates (February and May 2017) were compared, no significant differences was observed
159 in February but in May, the C.I. of mussels from Lannion were significantly higher than those
160 of Brest mussels (ANOVA, $p < 0.05$ and Tukey's *post hoc* test) (Fig. 5). There was no
161 significant difference between the two C.I. in February and in May for the same site and no
162 significant link appeared between observed mortality and C.I. (Pearson correlation, $p > 0.05$).
163



164

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



167
 168 Figure 5: Comparison of the Condition Indices (C.I.) of mussels from the 3 sites (Brest, Lannion, St. Brieuc) on
 169 both dates (February and May 2017). Values are means \pm SEM (ANOVA, * $p < 0.05$ and Tukey's *post hoc* test).
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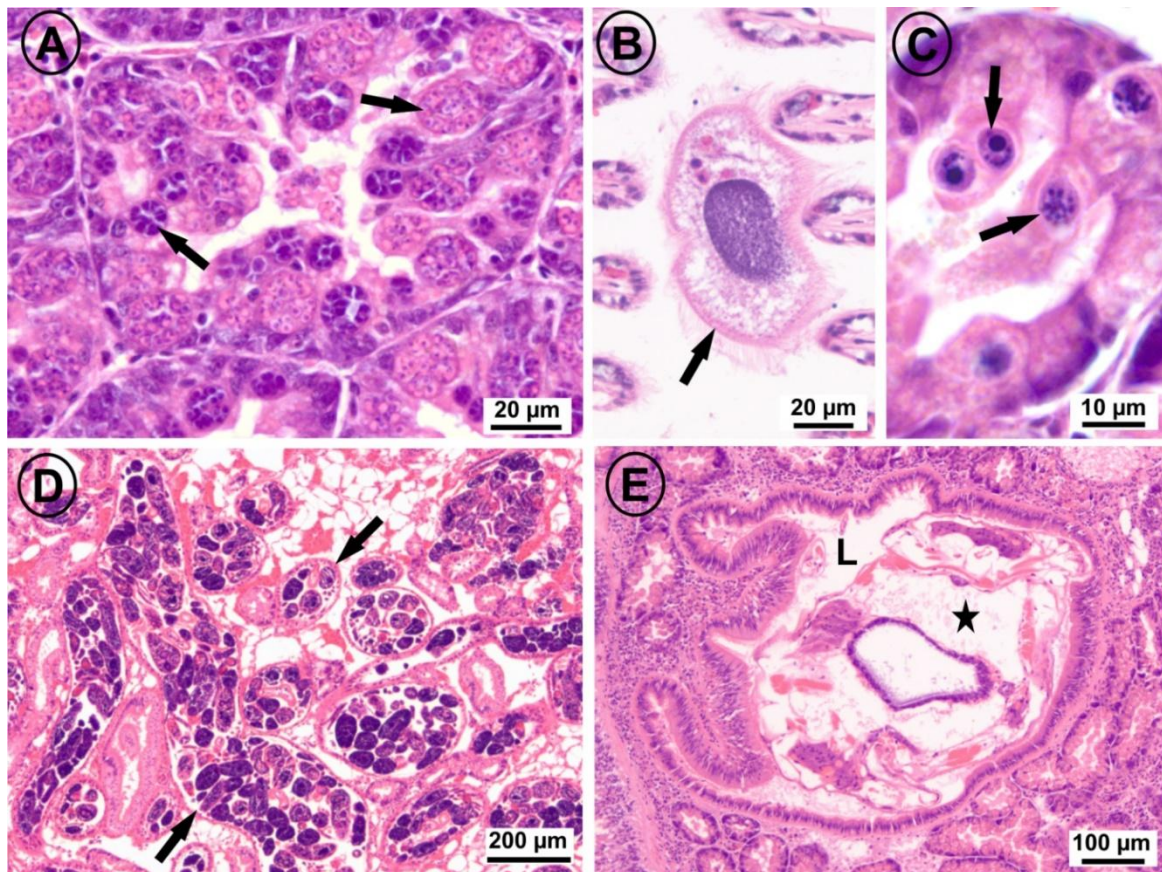
171 **3.3. Histological examination**

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

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

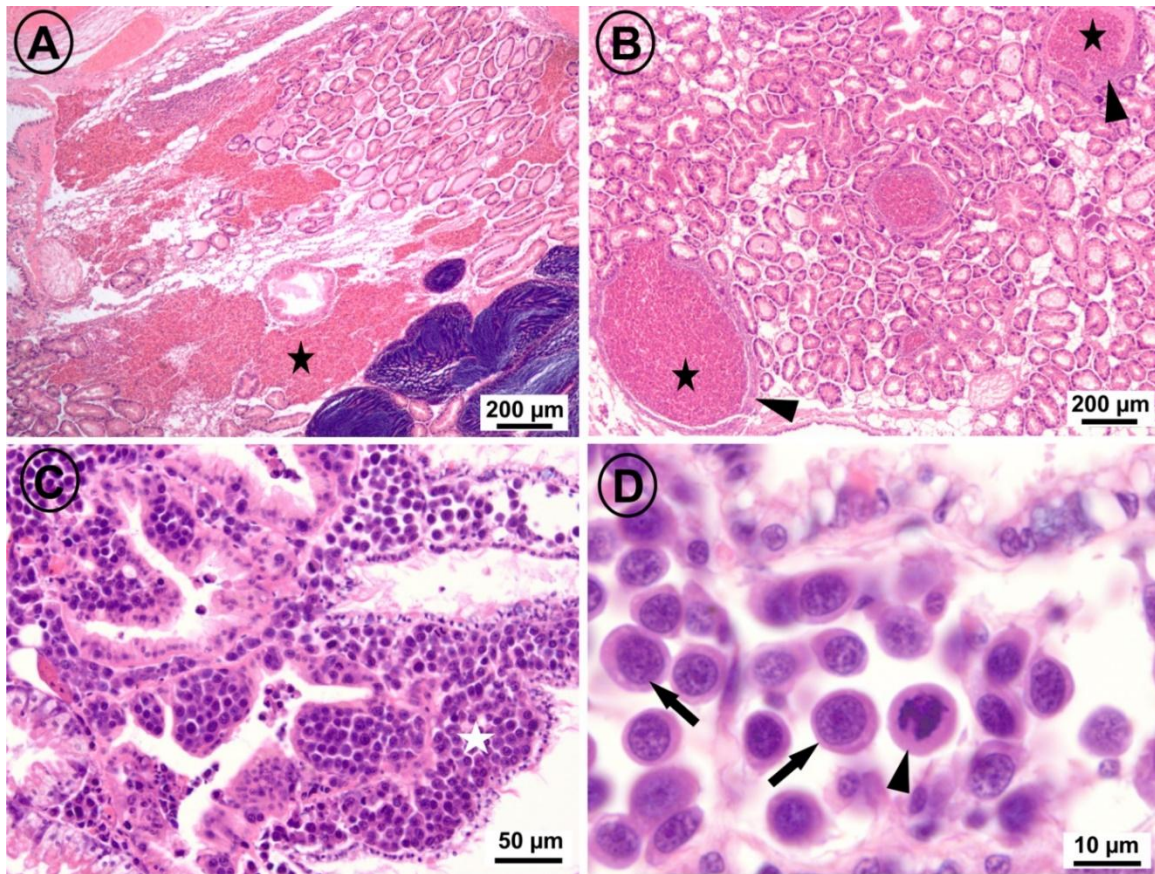
212 decrease from April to September. Furthermore, 18% of mussels from Brest showed
213 disseminated neoplasia in April, while it was not found in February or May, and its
214 prevalence was 2% in September. In Brest, the prevalence of most symbionts and the other
215 pathological were highest in April.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

222



223

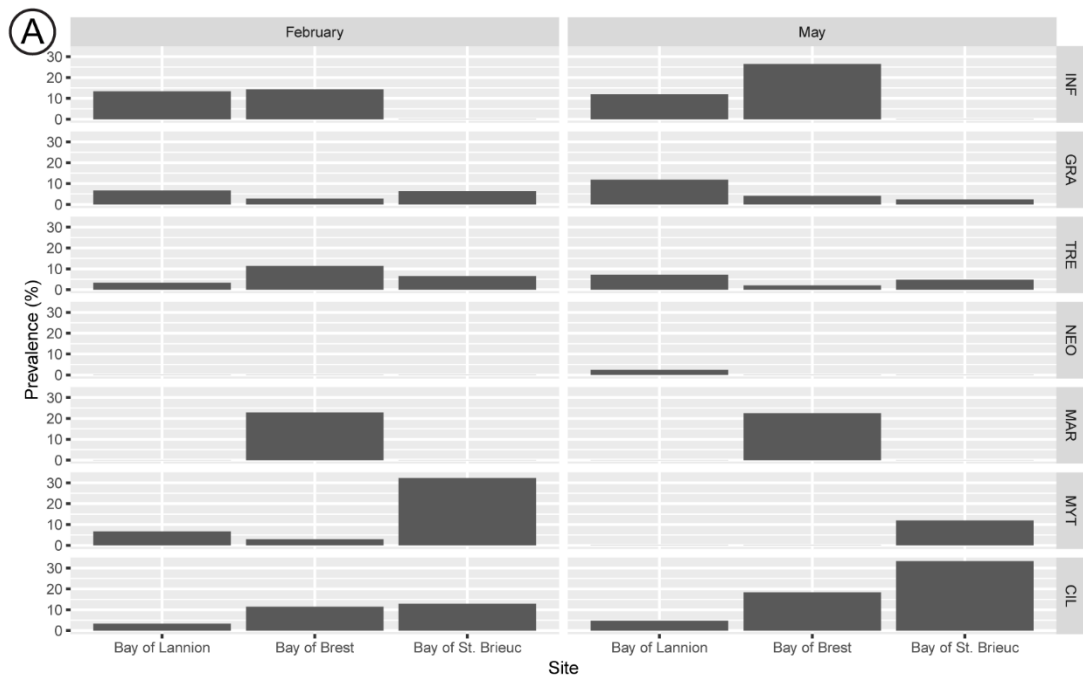
224 Figure 6: Micrographs of histological sections showing symbionts found in *Mytilus edulis*. A: section of the
225 digestive gland showing different developing stages of *Marteilia* sp. (arrows) in the epithelium of digestive
226 tubules. B: section through the gills showing a cell of the ciliate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



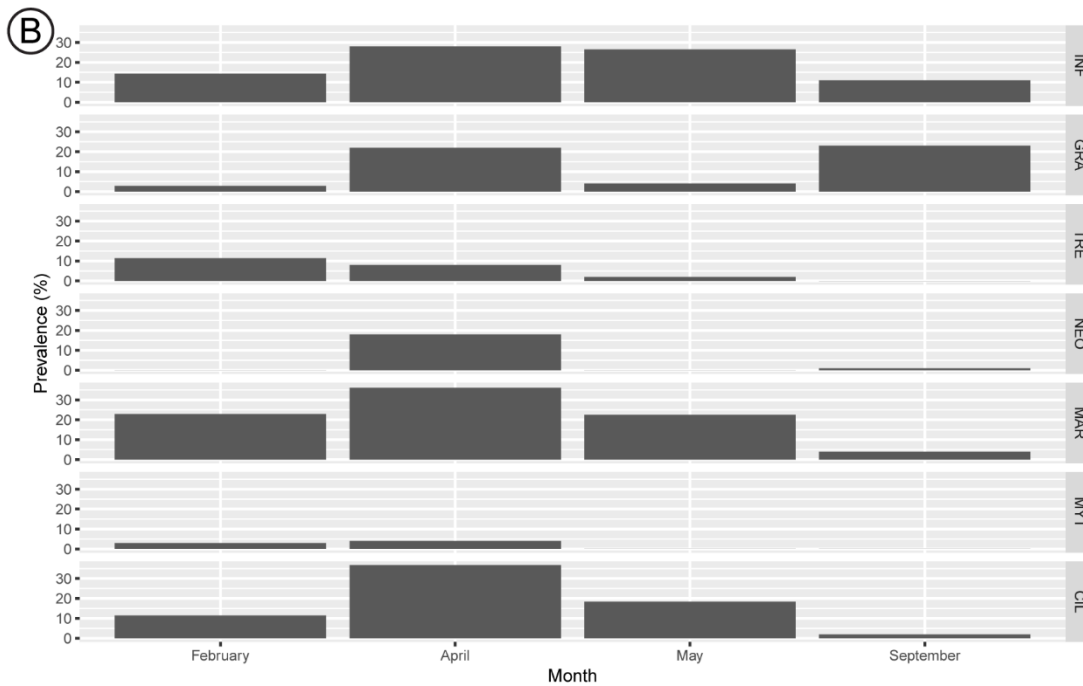
230

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

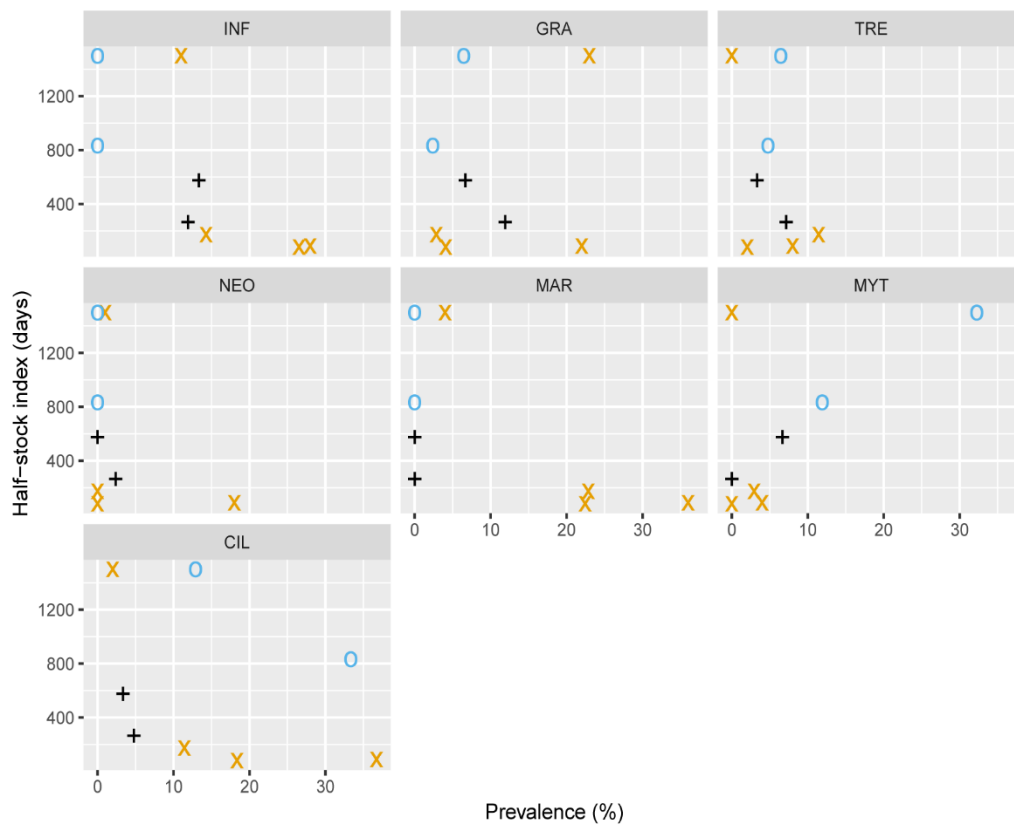
238



239



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



245

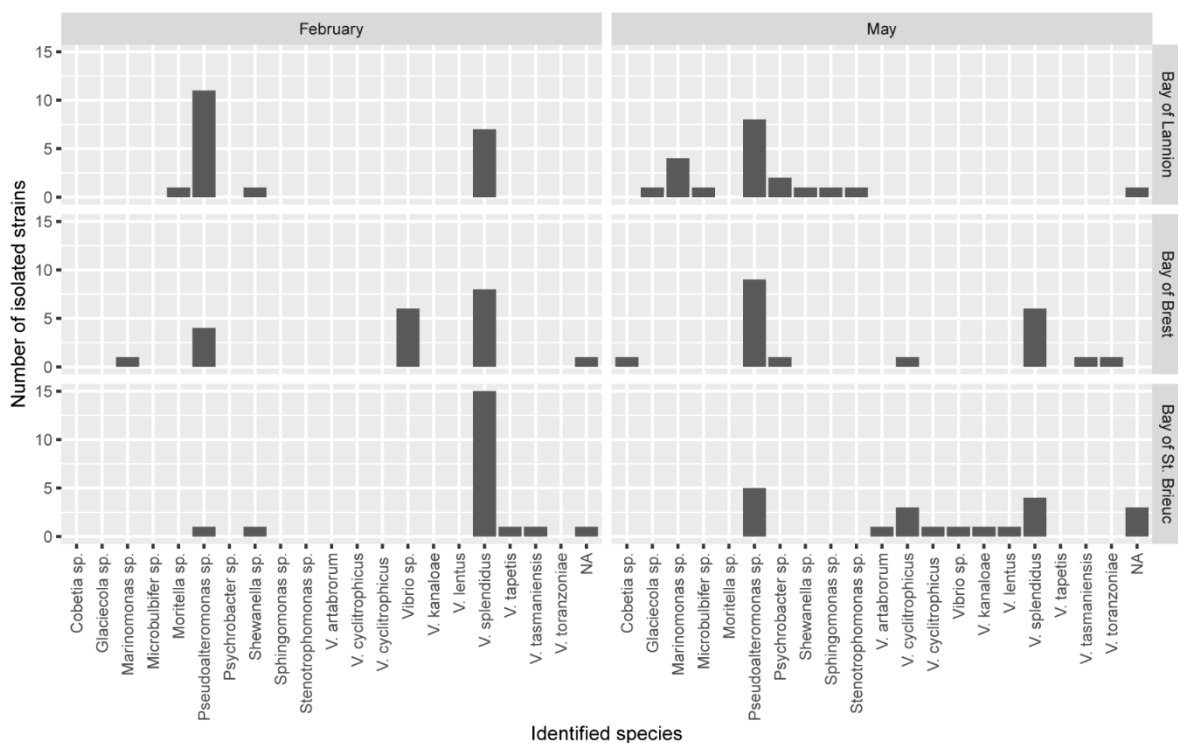
246 Figure 9: Link between the prevalence (%) of symbionts and histopathological conditions and the half-stock index (days) for each site: Brest (letter x), Lannion (plus sign) and St. Brieuc (open circle). INF: haemocytic
 247 infiltration; GRA: granulocytomas; TRE: trematode sporocysts enclosing cercariae; NEO: disseminated
 248 neoplasia; MAR: *Marteilia* sp.; MYT: *Mytilicola* sp.; CIL: ciliates.
 249
 250

251 3.4. Molecular analyses

252 3.4.1. Bacterial profiles

253 Following the identification of the 120 isolated bacterial strains from tissue culture, it was
 254 noted that bacterial diversity was relatively higher in May than in February (Fig. 10). In
 255 February, between four (Brest & Lannion) and five (St. Brieuc) different species per site were
 256 present among the 20 strains isolated compared to seven (Brest & St. Brieuc) and nine
 257 (Lannion) different species in May. However, despite this difference in diversity, two bacteria
 258 remained dominant: *V. splendidus* and *Pseudoalteromonas* sp. *V. splendidus* prevalence in
 259 February was 42% in Brest, 35% in Lannion and 80% in St. Brieuc; in May, prevalence was
 260 30% in Brest, 0% in Lannion and 23% in St. Brieuc. In February *Pseudoalteromonas* sp.
 261 prevalence was 21% in Brest, 55% in Lannion and 5% in St. Brieuc; while in May,
 262 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

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

282

283 ***3.5. Chemical analyses of flesh***

284 Table 2 shows trace element concentrations in the mussel tissues and the maximum level
285 (ML) set by the Commission Regulation ((EC) No. 1881/2006 amended by (EC) No.
286 629/2008 and (EC) No. 420/2011) for regulated chemical compounds in the relevant
287 foodstuffs category of bivalve molluscs. Comparing the three sites in February, mussels from
288 St. Brieuc had concentrations of chromium (Cr), molybdenum (Mo), nickel (Ni) and titanium
289 (Ti) overall 2-4x higher than those of Brest and Lannion. The Lannion site was distinguished
290 by iron (Fe) and aluminum (Al) concentrations that were more than 2x lower than the other
291 two sites. In contrast, lead (Pb) concentration was highest in Brest, more than 5x higher than
292 that of the mussels from Lannion and St. Brieuc, and exceeding the ML. Regarding temporal
293 variation of chemical compounds in Brest mussels, Pb concentration was similar in February
294 and April with a twofold decrease in September; Fe and Al concentrations showed noticeable
295 decrease over time. The highest concentrations were found in winter and a general decrease
296 was observed throughout the study period.

297

299 Table 2. Trace element concentrations (mg/kg w.w.) in mussel tissues collected from three bays in northern
 300 Brittany: Brest (BRE), Lannion (LAN) and St. Brieuc (STB), in February 2017, and in Brest in April and
 301 September 2017. Limit of detection: < 0.002 mg/kg). *ML: Maximum Level set by the Commission*
 302 *Regulation (EC) No. 1881/2006 amended by (EC) No. 629/2008 and (EC) No. 420/2011.*
 303

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

304

305

306 **4. Discussion**

307 An *in situ* study combining various analytical procedures was performed to explore
 308 causes of mussel mass mortality. The experimental design for field work involved assuming
 309 the culture procedures and using the mussel batches of the professional mussel farmers, taking
 310 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 Gouletquer (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

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

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

348

349 **4. 2 Mussel symbionts**

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

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

363 despite a high infestation intensity reported in English and Spanish mussels, it is evident that
364 the host population can sustain the infestation indefinitely (Davey, 1989; Robledo *et al.*,
365 1994). Again, our results were consistent because the highest prevalence of this copepod was
366 recorded in St. Brieuc and no abnormal mortalities were observed over the period at this site;
367 the mortality peak observed in May could be attributed to weakness due to the breeding
368 season, which could be intensified by the presence of *Mytilicola* sp. as observed by Brienne
369 (1964) and Blateau (1989).

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

378 No significant statistical link was established for *Marteilia* sp., but the p-value close to
379 0.05 should be highlighted as it was entirely influenced by results from Brest. The presence of
380 *Marteilia* sp. only in the most affected site should not be neglected, especially because it not
381 only severely harms flat oysters, *Ostrea edulis*, but also *M. edulis* and *M. galloprovincialis*
382 (Villalba *et al.* 1993; Villalba *et al.* 1997; Fuentes *et al.*, 1995; Arzul *et al.*, 2014). Indeed,
383 several studies have shown that high mortality rates observed in certain mussel populations in
384 Spain and France were positively correlated with the presence of this parasite (Villalba *et al.*,
385 1993; Garcia *et al.*, 2005). Marteiliosis leads to the arrest of growth due to the degradation of
386 digestive cells, loss of glycogen in tissues and resulting in considerable weight loss of the
387 mollusc (Grizel & Tigé, 1973). This disease could explain the lower C.I. in Brest.
388 Marteiliosis also causes a general weakening of the host, promoting opportunistic parasite

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

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

410

411 ***4.3 Lesions and alteration: hemocytic infiltration, granulocytomas and neoplasia***

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

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

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

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

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

465

466 **4.4 Chemical contamination**

467 First, and in relation to what has been previously discussed, when food availability is
468 low and competition between individuals is high, mussels increase their levels of water uptake
469 through their gills. This leads to congestion and accumulation of useless substances in their
470 tissues, including pollutants, which could result in increase of mortality rates (Dailianis,
471 2010). It is known that anthropogenic compounds and heavy metals play roles in defence
472 capacities of bivalve molluscs and could increase susceptibility to disease (Coles *et al.*, 1995;
473 Pipe & Coles, 1995; Morley, 2010). In addition, the effects of environmental contaminants
474 sometimes correspond to a direct toxic action on tissues or cells (Gagnaire *et al.*, 2004).

475 Some of the elements analyzed, Hg, As, Cd, Pb and Sn, have no known biological
476 function and, except for Sn, are included in the list of the ten chemicals of major public health
477 concern as part of International Programme on Chemical Safety (World Health Organisation,
478 2010). These elements are natural trace components of the aquatic environment, but their
479 levels increase due to agricultural, industrial and mining activities. Even low metal
480 concentrations may threaten the health of aquatic and terrestrial organisms, humans included
481 (Sarmiento *et al.*, 2011). Among all the metallic elements that Moschino *et al.*, (2016) have
482 measured in mussel tissues (As, Cd, Cr, Pb, Al, Fe, Hg, Cu, Ni, Zn) during an *in situ* study
483 over several years, a positive correlation between mussel mortality rate and Pb, Fe and Al was
484 observed. Furthermore, the average concentrations they measured in the mussel tissues for
485 these three elements were comparable with ours. It should also be noted that for Fe and Al,
486 relatively similar high concentrations were found in St. Brieuc and Brest, while for lead, only
487 mussels from Brest were above the threshold. Several biomarkers representative of the health
488 status of the aquatic environment have been identified in mussels (Depledge, 1994; Dailianis,
489 2010), and it was shown that mussels have lower defense mechanisms against metal oxidative
490 challenge and toxicity than oysters. Indeed, Funes *et al.* (2006) have shown that activities of
491 antioxidant enzymes are insufficient (compared to those observed in the Pacific oyster, *C.*
492 *gigas*), which means that mussels are not sufficiently protected from the oxidative stress

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

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

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

516

517 ***4.5 Bacterial profiles***

518 Species belonging to the genus *Pseudoalteromonas* are widely distributed in marine
519 environments globally. Some studies have shown a positive role of *Pseudoalteromonas* sp.
520 biofilms in the settlement of mussel, *Mytilus coruscus* larvae (Yang *et al.*, 2013; Li *et al.*,
521 2014). On the other hand, Venkateswaran & Dohmoto (2000) identified a species of
522 *Pseudoalteromonas* which play a natural antifouling role due to the products it excretes,
523 which prevent fixation of mussel byssal threads. However, no pathogenic or lethal role has
524 been demonstrated by this species on bivalves. Species of the genus *Pseudoalteromonas* and
525 *Vibrio* are part of the normal microflora of temperate water marine animals and their
526 proportion increases during the spoilage of the bivalves (Gram & Huss, 1996; Madigan *et al.*,
527 2014). Also, Lokmer & Wegner (2015) showed that host-associated microbial communities
528 are linked to abiotic and biotic factors.

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

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

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

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

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

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

579

580 **5. Conclusion**

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

589

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1052

1053 **Additional file 1: Supplementary Materials and Methods**

1054 *PCR screening for bivalve mollusc known pathogens*

1055 For each mussel, a pool of minced tissues from foot, adductor muscle, gills, mantle, and
1056 digestive gland was subjected to DNA extraction using a QIAamp DNA minikit[®] (Qiagen,
1057 Courtaboeuf, France) following the manufacturer's protocol for blood or body fluids, except
1058 for elution performed in 60 µL Qiagen elution buffer AE. The quality of the extracted DNA
1059 was checked with NanoDrop[™] 2000c spectrophotometer (ThermoFisher Scientific[™],
1060 Waltham, MA USA). Within the same batch, the individual extracts were pooled in groups of
1061 5 to have 4 replicates per batch for each PCR done. The known bivalve pathogens
1062 investigated by TaqMan[®] real-time PCR and classical PCR were: *Bonamia* spp.,
1063 *Haplosporidium nelsoni*, *Marteilia* sp., *Mikrocytos mackini*, *Nocardia crassostreae*, Ostreid
1064 Herpesvirus type 1 (OsHV-1), *V. aestuarianus*, *V. tubiashii*, *V. harveyi*, and *V. splendidus* (the
1065 true species *V. splendidus* of the Splendidus clade named here Splendidus cluster) (Table S1).
1066 Amplification reactions were performed in a total volume of 25 µL using a SmartCycler[®]
1067 (Cepheid, USA). Each reaction contained 12,5 µL of a Premix Ex Taq[®] 2 X Takara[®] (Lonza,
1068 Verviers, Belgium), 9 µL of purified water, 2 µL of DNA sample (replaced with 2 µL of
1069 purified water in the negative control) and 0.5 µL of each primer (20 µM) and probe (10 µM)
1070 or SYBR Green (for *N. crassostreae*). Each assay included negative and positive control
1071 reactions. The thermal cycle profile consisted of 95 °C for 10 s followed by 40 cycles of 95
1072 °C for 5 s and 60° for 30 sec (or 62 °C for *V. splendidus* or 64 °C for *V. harveyi*). Beforehand,
1073 for each real-time PCR developed for this study, the inclusivity and exclusivity were tested.
1074 Moreover, in order to exclude false-positive results, when a signal was obtained, the amplicon
1075 was sent to Eurofins MWG Operon (Ebersberg, Germany) to be purified and subsequently
1076 sequenced; only then, after using the NCBI BLAST, confirmation of the targeted pathogen
1077 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
<i>Bonamia</i> spp.	28S rRNA	Fw TCCCTGCCCTTTGTACACA Rv CTCTTATCCACCTAATTCACCTCAG Pr TxR-CGCCCGTCGCTTCTACCGATT-BHQ2	Present paper
<i>Haplosporidium nelsoni</i>	SrRNA	Fw CACGCGCGCTACAATGT Rv CGAGATTACCCGGCCTTCT Pr FAM-CACGCAACGAGTTCAACCTTGCC-BHQ1	Present paper
<i>Marteilia</i> sp.	ITS1	Fw CACTACTCTTCGCTTTTCGAT Rv GACTACCCGTGCCGAACA Pr Cy3-TCGCAAACAGGAAGCGGCTCTC-BHQ1	Present paper
<i>Mikrocytos mackini</i>	28S rRNA	Fw GGTGGCCGAATGACGTAGT Rv GCCTATGACAGCACGAAGCA Pr Cy5-CCGCTTCGGCGTGCAGTCTC-BHQ2	Present paper
<i>Nocardia crassostreae</i>	16S-23S rNA ITS	Fw CCTCGATACCGCCGAAGAA Rv CAACACACCCGCATCAA	Carrasco <i>et al.</i> (2013)
OsHV-1	B region	Fw GTCGCATCTTTGGATTAAACAA Rv ACTGGGATCCGACTGACAAC Pr TxR-TGCCCTGTCATCTTGAGGTATAGACAATC-BHQ2	Martenot <i>et al.</i> (2010)
<i>V. aestuarianus</i>	dnaJ	Fw GTATGAAATTTTAACTGACCCACAA Rv CAATTTCTTTCGAACAACCAC Pr TxR-TGGTAGCGCAGACTTCGGCGAC-BHQ2	Saulnier <i>et al.</i> (2009)
<i>V. tubiashii</i>	vtpA	Fw GGTACGGACTATCCGGGATT Rv TTCACCGCTGAGTTGTTTCAT Pr Cy3-ATCGTCGATAAATCAGGCACAACCTGT-BHQ1	Present paper
	vth	Fw CGGTTGATATTCGCGTCAA Rv GTGTGAAACCCTGCGAAGTA Pr Cy5-TATCACAGATGCGCTCGGTTTCAGTC-BHQ2	Present paper
<i>V. harveyi</i>	16S rRNA	Fw CGAGCGGAAACGAGTTATCTG Rv CTCACCAACTAGCTAATCCACCTA Pr TxR-CCGCATAATACCTACGGGTCAAAGAGGG-BHQ2	Present paper
<i>V. splendidus</i> (<i>Splendidus</i> cluster)	toxR	Fw AGCAGCGGCTGAAATTGCA Rv GGCCGCAGTTGGTGTGTT Pr FAM-CAATGACTGAAGCTGTTCGAGCCC-BHQ1	Oden <i>et al.</i> (2018)