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# 1 Implementation of various approaches to study the prevalence, incidence 2 and progression of disseminated neoplasia in mussel stocks

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11

## 12Abstract

13Marine mussel production is of substantial economic interest in numerous coastal areas worldwide,  
14making crucial the study of pathologies that affect them. Disseminated neoplasia (DN) has recently  
15been suggested to be linked to blue mussel, *Mytilus edulis*, mortality outbreaks observed in France  
16since 2014, although the evidence remains indirect. In order to improve DN detection and monitoring,  
17we compared the sensitivity of four diagnostic tools, namely haemocytology, histology, flow  
18cytometry, and genetics. Haemocytological examination gave the best results in sensitivity and had the  
19advantage of being non-invasive, allowing disease progression to be followed in affected mussels.  
20Using this approach, we showed that DN progression is usually slow, and we provide evidence of  
21remission events. We observed a high diversity of forms and mitotic features of neoplastic cells  
22located in the vesicular connective tissue but rarely in the haemolymph. Circulating cells occur as four  
23main types but are homogenous in morphology and DNA content within a single individual.  
24Polyploidy proved very high, from 8N to 18N. Genetic analysis of haemolymph DNA showed that a  
25*Mytilus trossulus* genetic signal was associated with almost all the DN cases here diagnosed by  
26haemocytological examination, regardless of the DN type. This result corroborates DN is a  
27transmissible cancer that first originated in a *M. trossulus* host and subsequently crossed into *M.*  
28*edulis*. No pre-neoplastic conditions were detectable. The prevalence of the disease was quite low,

29which, together with the low morbidity observed in the lab, suggest DN is unlikely to be the direct  
30cause of mortality outbreaks in France.

31

32**Keywords:** Blue mussel, Genetic chimerism, Genomic abnormalities, Haemolymph cytology,  
33Neoplasia, Trossulus

34

### 351. Introduction

36Disseminated neoplasia (DN) has been reported in several species of bivalves belonging to various  
37families, including *Cardiidae*, *Myidae*, *Mytilidae*, *Ostreidae*, *Tellinidae*, and *Veneridae* (Farley, 1969a;  
38Bayne, 1976; Balouet et al., 1978; Auffret and Poder, 1986; Elston et al., 1992; Barber, 2004; Carballal  
39et al., 2015), with a world-wide distribution. Within the *Mytilidae* family, the disease has been  
40described in blue (*Mytilus edulis*), Mediterranean (*Mytilus galloprovincialis*), and Pacific (*Mytilus*  
41*trossulus*) mussels (Farley, 1969b; Green and Alderman, 1983; Moore et al., 1991; Elston et al., 1992;  
42Galimany and Sunila, 2008; Carella et al., 2013; Gombač et al., 2013). The DN condition is  
43characterised by the presence of circulating anomalous enlarged basophilic cells (Lowe and Moore,  
441978; Green and Alderman, 1983) and by their infiltration of hemal spaces of all organs, with the loss  
45of vesicular connective tissue in severe cases. These round-shaped cells display enlarged  
46heteromorphic and sometimes multiple nuclei with prominent nucleoli, and high nucleus to cell  
47diameter ratio (Farley and Spark, 1970; Lowe and Moore, 1978; Mix et al., 1979). Anomalies in DNA  
48content and ploidy in neoplastic bivalve cells have been reported in many studies (Lowe and Moore,  
491978; Elston et al., 1990; González-Tizón et al., 2000; da Silva et al., 2005; AboElkhair et al., 2009;  
50Le Grand et al., 2010; Díaz et al., 2013; Vassilenko and Baldwin, 2014; Carella et al., 2017).

51Various aetiological origins have been suggested. Exposure to pollutants and sublethal levels of  
52biotoxins were suspected to be involved in DN development (Lowe and Moore, 1978; Reinisch et al.,  
531984; Landsberg, 1996) but was not confirmed in subsequent studies (Green and Alderman, 1983). On  
54the other hand, the transmission of the disease between mollusc individuals including mussels, through

55cohabitation or haemolymph injection, has been proven (Elston et al., 1988; Twomey and Mulcahy,  
561988; Kent et al., 1991; House, 1997; Weinberg et al., 1997), indicating that an infective agent, or the  
57neoplastic cells themselves, could be involved. DN transmission has been effected by injection of  
58neoplastic whole cell preparations, but when cell-free 0.45 µm filtered preparations were used,  
59conclusions by different researchers on the success of the disease transmission were not unanimous  
60(McLaughlin et al., 1992; House et al., 1998; Collins and Mulcahy, 2003).

61Reverse transcriptase activity has been reported in DN-affected bivalves (House et al., 1998; Romalde  
62et al., 2007; AboElkhair et al., 2009) and some studies have highlighted the presence of a retrovirus-  
63like agent that could readily produce the chromosomal aberrations associated with DN and possesses a  
64reverse transcriptase (Oprandy et al., 1981; Oprandy and Chang, 1983; Romalde et al., 2007).  
65However, in most cases, no infectious agent was confirmed and AboElkhair et al. (2012) did not  
66provide clear evidence of retrovirus involvement in the disease. Recent studies (Metzger et al., 2015;  
67Metzger et al., 2016) showed that DN can sometimes be a contagious form of neoplasia. Transmissible  
68cancers, evidenced by intra- or inter-species genetic chimerism, have recently been identified in  
69various marine bivalves (Metzger et al., 2016), including mussels. Riquet et al. (2017) identified  
70genetic chimerism in some French *M. edulis* individuals that carried *M. trossulus* alleles at diagnostic  
71markers even though this species is not present in France, suggesting that this could be related to the  
72contagious cancer described by Metzger et al. (2016).

73Mussel farming is of great economic importance in several European countries. French production is  
74represented by two species, *M. edulis* and *M. galloprovincialis*, which are able to hybridise in several  
75areas where they coexist (Bierne et al., 2003). Production in France amounts to around 75.000 tons  
76each year (FAO, 2016). However, since 2014, mass mortality events (90-100%) have occurred in  
77France, beginning in the Pertuis Charentais (Nouvelle-Aquitaine, France) (Béchemin et al., 2015) and,  
78since 2016, in other farming areas in Brittany (France) (Bernard and Allain, 2017, Bernard et al., 2017;  
792018). The causes of these mortality events are still unresolved. Some studies hypothesized the  
80involvement of *Vibrio* species (Ben Cheikh et al., 2016, 2017; Oden et al., 2016), probably coupled  
81with stressful environmental conditions (Polsenaere et al., 2017). Benabdelmouna and Ledu (2016)

82 associated abnormal outbreaks of mussel mass mortality with poor cytogenetic quality, particularly in  
83 terms of ploidy variations in circulating cells. In a second study, Benabdelmouna et al. (2018) linked  
84 these genomic abnormalities to ongoing neoplastic processes. DN was also suspected to be involved in  
85 these events after the finding of a high prevalence of DN in a *M. edulis* collection from Lannion  
86 (France) affected by mortality in October 2016 (Burioli et al., 2017). Massive mortalities of  
87 economically important bivalve stocks have occasionally been linked to high prevalence of DN in  
88 other parts of the world (Frierman and Andrews, 1976; Elston et al., 1992; McGladdery et al., 2001;  
89 Villalba, et al., 2001).

90 We studied the prevalence of DN in various mussel stocks, some of them regularly affected by  
91 mortality events. Because we wanted to maintain our live mussel specimens to follow the incidence  
92 and progression of the disease in the lab, we tested the sensitivity of two non-invasive methods, based  
93 on cytology and flow cytometry of haemolymph. On sub-samples, we compared the results of these  
94 two methods with histological observation and genetic analysis of haemolymph DNA to diagnose the  
95 DN state. Finally, we used cytology to follow DN progression in five mussel groups over 4 to 7  
96 months.

97

## 982. Materials and Methods

### 992.1. Sampling

100 Eighteen batches of mussels were collected during sampling campaigns conducted between July 2017  
101 and June 2018. A total of 2,188 mussels were collected from six farming sites and a wild population  
102 (Table 1 and Figure 1). We also reported the origin areas where mussel spat was collected from the  
103 wild in the prior year before being moved to their final growing sites. At time of sampling, individuals  
104 from 12 batches were more than 1 year in age, while the others were aged approximately 1 year or less  
105 (the age of wild individuals is unknown). Sampled mussels were farmed on the intertidal zone (Agon-  
106 Coutainville, St Brieuc, Daoulas, and Pénestin) or in suspension in deep waters (Lannion and  
107 Camaret). Wild mussels (Roscanvel) were collected on the intertidal zone. Sixteen batches were

108previously identified as mainly *M. edulis* and two as mainly *M. galloprovincialis* according to  
109morphological characters and by genotyping the repetitive region of foot protein 1 in 10 individuals  
110per batch according to Inoue et al. (1995).

111A monitoring program for mussel mortality was conducted in Northern Brittany during 2017 and 2018  
112(Bernard et al., 2017, 2018), in the sites of St Brieuc, Lannion, and Daoulas. The station of Daoulas  
113was affected by heavy mortality between March and June 2017 ( $\geq 65\%$  cumulative mortality rate of  
114the period) and during May 2018 (about 30%). In Lannion, the phenomenon was weaker but lasted at  
115least until July 2017 with  $\geq 35\%$  cumulative mortality. Unfortunately, this population was not  
116monitored thereafter. No anomalous mortality was observed in St Brieuc. The sites of Roscanvel,  
117Camaret, and Pénestin were not included in this monitoring program, thus no official information on  
118putative mortality is available for these populations. However, the farmer at Camaret reported  
119cumulative mortality ranging between 60% and 80% in 2017. In Normandy, according to the French  
120network for the monitoring of mollusc health REPAMO ([https://wwz.ifremer.fr/sante\\_mollusques/](https://wwz.ifremer.fr/sante_mollusques/)), a  
121mortality event started in Agon-Coutainville in June 2018.

122

### 1232.2. *Evaluation of the various diagnostic methods*

124Because we wanted to maintain mussels alive with the aim to study the incidence of DN and the  
125progression of the disease, we tested the non-invasiveness of regular haemolymph drawing by  
126comparing the mortality of sampled and naïve individuals. Mortality was monitored daily for 2 months  
127in four mussel batches (N=791) (see [Table 1](#)). Cumulative mortality rates were compared for  
128individuals from which 100  $\mu$ L of haemolymph was drawn every 3 weeks and those not subjected to  
129haemolymph drawing (naïve) (see [Table 2](#)). Once the mussel batches arrived at the LABÉO  
130laboratory, the groups used for this trial were subdivided and held in 10-L tanks, a maximum of 100  
131individuals per tank, in natural sea water maintained at 15°C, under oxygenation conditions. Complete  
132water changes were done on alternate days.

133 Haemocytological observation was conducted on all batches on their arrival at the laboratory. The  
134 sensitivities of haemocytological observation and flow cytometry were then compared and  
135 accompanied by histological observation and genetic analysis in some batches (see Table 1). A total of  
136 331 individuals underwent haemolymph drawing to allow both cytological observation and flow  
137 cytometry. We used a neoplastic individual from Batch 2 to implement the flow cytometry protocol.  
138 All individuals from the Batches 10, 12, and 13 (N=241) were simultaneously subjected to both  
139 analyses on their arrival; flow cytometry was not immediately carried out for Batch 11, but on  
140 survivors in February alongside haemocytological observation; for Batches 2 and 5, flow cytometry  
141 was carried out on randomly sampled individuals. Histological observation is an invasive method.  
142 Thus, when the batch was kept in tank for a long period of time in order to study the DN incidence and  
143 disease progression in affected mussels (Batches 2, 4, 5, and 8), histology was carried out on each  
144 moribund individual (along with haemocytological observation) and on half of the survivors at the end  
145 of the monitoring period (402 individuals in total). Genetic analysis was conducted on 114 individuals  
146 also subjected to haemocytological observation, flow cytometry, and histology. The various protocols  
147 used are reported below.

### 148 2.3. *Haemocytological examination*

149 The mussels were anaesthetised by bathing them for 30 min in a solution containing 50 g/L of  
150 magnesium chloride dissolved in 2:1 distilled water:sea water (Suquet et al., 2009). Haemolymph (40  
151  $\mu$ l) was withdrawn from the adductor muscle with a 1-mL sterile syringe fitted with a 27-gauge needle  
152 and refrigerated to avoid aggregation. After addition of 160  $\mu$ L of artificial seawater, the haemolymph  
153 solution was “cytopun” (Shandon™ Cytospin™ 4, Thermo Scientific™; 10 min, 800 rpm) on coated  
154 slides (Shandon™ Cytoslides™, Thermo Scientific™), fixed, stained with May-Grünwald Giemsa,  
155 and observed under light microscopy (100X, 500X).

156 The entire area of the cell spot on the cytoslide was scanned in all samples to search for neoplastic  
157 cells, which are characterised by an enlarged diameter, elevated nucleus/cell diameter ratio (NCR), and  
158 high basophily. Four developmental stages of DN were differentiated according to the proportion of  
159 neoplastic cells in haemolymph: “non-diseased” when no neoplastic cells were observed, “low

160intensity” (less than 20% of neoplastic cells), “moderate intensity” (20% to 75%), and “high intensity”  
 161(more than 75%).

162In order to determine if a pre-neoplastic stage could be detected in the individuals before the  
 163appearance of the first typical neoplastic cells, the various haemocyte populations were then also  
 164characterised in terms of morphology and relative proportions. The mussel conditions showing  
 165atypical haemocytograms in terms relative percentage of cell populations according to statistical  
 166analysis were considered to be affected by “haemocytic disorders”. The cell and nucleus diameters of  
 167each haemocyte type were then measured. Cell and nuclear diameters were determined in 50 cells per  
 168cell type (when there were sufficient numbers), in 10 mussels/batch. The disorder-affected individuals  
 169were initially excluded from this data collection carried out to determine the normal condition.  
 170Nevertheless, in order to better characterise these anomalies, 50 cells per cell type in 1 to 3 atypical  
 171mussels per batch were measured. Finally, neoplastic cells were described in the same way for all  
 172affected mussels, with 1 to 30 neoplastic cells measured depending on the number of anomalous cells  
 173in the haemolymph.

174The total haemocyte count was performed directly from cytological stained slides made from 30  
 175individuals in each of the 18 batches, excluding initially the individuals affected by DN and  
 176“haemocytic disorders”. Haemocytes were counted in four random microscope fields at 500X  
 177magnification. Haemocyte densities per millilitre of haemolymph were estimated as follows:

$$\frac{\text{No. haemocytes}}{\text{mL haemolymph}} = \frac{\Sigma \text{ haemocytes counted}}{\text{No. fields counted}} \times \frac{\text{area of spot on cytoslide (mm}^2\text{)}}{\text{area of microscope field (mm}^2\text{)}} \times \frac{1}{\text{volume of haemolymph drawn (mL)}}$$

$$= \frac{\Sigma \text{ haemocytes counted}}{4} \times \frac{28,27}{0,125} \times 25$$

178

179Then, total haemocyte count was also determined in mussels affected by neoplasia and “haemocytic  
 180disorders”.

#### 1812.4. *Flow cytometry analysis*



182When flow cytometry analysis was conducted on mussel individuals at the same time as cytological  
183analysis, a supplementary volume of 100  $\mu$ L haemolymph was drawn and placed in a 1.5-mL  
184microtube; 500  $\mu$ L of ethanol 100% was added to fix the cells, prevent aggregation, and increase cell  
185permeability. This suspension was stored at  $-20^{\circ}$  C until the next steps of the process. First, each  
186aliquot of haemolymph was centrifuged at 600x g for 5 min and the pellet was resuspended in 500  $\mu$ L  
187sterile artificial seawater (1 L of reverse osmosis purified water, 23 g of sodium chloride, 1.49 g of  
188potassium chloride, 0.3 g of calcium chloride, and 1.23 g of magnesium sulfate heptahydrate) for 15  
189min at room temperature. A second centrifugation at 600 x g for 5 min was carried out and the pellet  
190was treated in 500  $\mu$ L of propidium iodide (PI) solution: PI (Life technologies) 0.05 mg/mL; RNase A  
191(Life technologies) 0.02 mg/mL; PBS (Eurobio 1X). Cells were stored for 45 min in the dark at room  
192temperature. Haemocytes were then analysed by flow cytometry using a Cytoflex (Beckman Coulter)  
193equipped with a laser providing 50 mW at wavelength 488 nm. For each individual, 10,000 cells were  
194analysed at a medium flow rate (30  $\mu$ L/min) to collect multiparametric data. The measurement of  
195DNA contents in haemolymph cells was based on the properties of PI, which intercalate into nucleic  
196acid molecules. When PI binds to nucleic acids its fluorescence intensity is proportional to the DNA  
197cell content, thus allowing the study of cell ploidy (Krishan, 1975). Because some cell populations had  
198extraordinarily high ploidy levels, each sample was run twice with two different gain settings. In order  
199to remove doublets from single cells, the IPBLUE-height *versus* IPBLUE-area dot-plot was used and a  
200region comprising single cells was drawn on this representation (adapted from da Silva et al., 2005),  
201doublets being excluded on the right of the selected area. The cells included in this region were then  
202plotted on a histogram in linear scale, used to calculate the percentage of cell populations according to  
203their DNA content. In particular, the percentages of cells in the normal diploid  $G_0/G_1$  and  $S/G_2/M$   
204phases were estimated according to Delaporte et al. (2008) and Benabdelmouna and Ledu (2016).

#### 2052.5. *Histological examination*

206A standard section of the mussel body,  $\sim$ 5 mm thick, containing the digestive gland, gut, mantle, gills,  
207and foot was fixed for 48 hrs in Davidson's fixative. Fixed tissues were then processed for routine  
208histological examination and 4- $\mu$ m thick sections were stained with Harris's haematoxylin and eosin

209(H&E). Histological sections were examined under a light microscope for the presence of abnormal  
210cells related to neoplasia.

#### 2112.6. *Genetics*

212DNA was extracted from the haemolymph using a NucleoMag 96 Tissue kit (Macherey-Nagel) and a  
213KingFisher™ Flex (ThermoFisher Scientific). Following the study of Riquet et al. (2017) we used  
214SNP markers diagnostic between *M. trossulus* and *M. edulis/M. galloprovincialis*. We used 12 SNPs  
215(supplementary Table S2) described in Simon et al. (2018). These markers were identified as being  
216diagnostic (fixed for alternative alleles in *M. trossulus* and *M. edulis/M. galloprovincialis*) in the  
217dataset of Fraïsse et al. (2016) and were subsequently verified to remain diagnostic with larger sample  
218sizes (Simon et al., unpublished). Non-hybrid individuals can nonetheless occasionally be  
219heterozygous at one or two of the 12 loci because incomplete lineage sorting and introgression is  
220pervasive in the *M. edulis* complex of species. Genotyping was subcontracted to LGC-genomics and  
221performed with the KASP™ chemistry (Kompetitive Allele Specific PCR, Semagn et al., 2014). The  
222results are a combination of two fluorescence values, one for allele 1 (X), and another for allele 2 (Y).  
223Following Cuenca et al. (2013) we transformed the data to produce a single measure of the relative  
224fluorescence of the two alleles, from 0 when the fluorescence of allele 1 dominates, to 1 when the  
225fluorescence of allele 2 (*i.e.* the *trossulus* allele) dominates, using the following formula:  $y' = Y/(X+Y)$ .  
226The fluorescence of the *trossulus* allele was averaged over the 12 loci to obtain a compound  
227multilocus estimate.

#### 2282.7. *Determination of DN prevalence, incidence and progression*

229The occurrence of DN in mussels was described in terms of prevalence, defined as the number of  
230mussels presenting anomalous cells in 100 individuals investigated by cytological examination. We  
231determined the initial DN prevalence in each of the 18 batches and the developmental stage of the  
232disease in affected animals.

233The incidence of the disease, defined as the number of new cases observed in survivors between two  
234consecutive haemolymph drawings expressed in percentage, and its progression in affected individuals

235 were evaluated in five batches (2, 4, 5, 8, and 11). Individuals were maintained in six different 10-L  
236 tanks (two tanks were used for Batch 11), in natural sea water maintained at around 15° C, under  
237 oxygenation conditions. Complete water changes were done on alternate days. Haemolymph drawings  
238 and haemocytological observations were carried out on all specimens, each month, for 4 to 7 months  
239 according to the batch (Table 1). In individuals that developed DN during the monitoring, a  
240 retrospective observation of haemocytological slides was carried out in search of haemocytogram  
241 modifications or changes in cell morphology that could represent a pre-neoplastic stage.

#### 2422.8. *Statistical analysis*

243 At the end of the trial for evaluation of the invasiveness of haemolymph drawing, the significance of  
244 the differences in mortality rate between the groups was evaluated using a chi-square test. Data from  
245 morphological characterisation (cell diameter, nucleus diameter and NCR), relative percentage of cell  
246 populations and total haemocyte count, determined by haemocytological observation, were analysed  
247 statistically to determine the significance of the inter-individual and inter-site differences. An  
248 Anderson-Darling test for normality was initially performed. If the data were found to follow a normal  
249 distribution, they were analysed using analysis of variance (ANOVA) followed by Tukey's post-hoc  
250 test on mean values. A Kruskal-Wallis test, followed by the ad-hoc multiple pair comparison test  
251 Kruskalmc (package pgirmess), were used on results requiring nonparametric analysis. Individuals  
252 showing significant differences were considered to be affected by haemocytic disorders.

253 Potential correlation between haemocytic anomalies and minority cells was evaluated by the  
254 calculation of the Pearson correlation coefficient. The same inferential statistic tests were used to  
255 compare the percentage of cells in the various peaks of ploidy during flow cytometry. Significance of  
256 the differences in the initial DN prevalence observed between the farming techniques (with and  
257 without tide exposure) and between the two age categories (>1 year and ≤1 year) was estimated  
258 through a chi-square test.

259 Statistical analysis was performed using R software, version 3.3.1. Results with a p-value <0.05 were  
260 considered statistically significant.

261

## 2623. Results

### 2633.1. *Haemolymph drawing is non-invasive*

264At the end of the trial to assess the invasiveness of the haemolymph drawing (2 months duration),  
265cumulative mortality in the different batches ranged between 12.0% and 38.2% (Table 2). No  
266significant differences were observed between control individuals and the individuals for which  
267haemolymph was drawn in each batch. The haemolymph drawing method was, thus, confirmed to be  
268non-invasive.

269

### 2703.2. *Haemocytological observations*

271In DN-unaffected individuals, cytological examination confirmed the presence of two main cellular  
272types, eosinophilic granulocytes (Figure 2A) and agranulocytes (Figure 2B and 2C). Granulocytes  
273were characterised by the presence of abundant brick-red granules in a beige cytoplasm, which varied  
274in number and size. The nuclei were typically spherical and occasionally ovoid and eccentric. A small  
275number of granulocytes were observed to contain two or more nuclei (Figure 2D). Lucent vesicles,  
276consistent with phagosomes, were sometimes observed in these cells. The cytoplasm of agranulocytes  
277appeared bluish and no granules were present. However, this population appeared more  
278heterogeneous, in terms of size, nucleus/cell diameter ratio (NCR), basophily, and nucleus shape. We  
279observed highly basophilic small cells (Figure 2B) with a very high NCR and larger cells,  
280characterised by a light-blue cytoplasm and a lower NCR with some cells showing a U-shaped nucleus  
281(Figure 2C). In some individuals, very pale red or blue vesicle coloration was observed in a few cells.  
282The results of the morphometric analysis carried out on cell and nucleus diameters and the NCR  
283values are reported in Table 3. Data collected from granulocytes showed a normal distribution while  
284those from agranulocytes did not. According to both parametric (ANOVA) and nonparametric  
285(Kruskal-Wallis) tests, no significant differences were observed among inter-individual and inter-site  
286measurements. From the first haemolymph drawing, we observed the presence of a few basophilic

287granulocytes (Figure 2E), smaller than eosinophilic granulocytes, characterised by numerous small  
288blue granules in the cytoplasm in about 1.90% mussels (Table 4). When present, these cells  
289represented less than 1% of the total haemocyte population. They were consistently present in the  
290batches from Daoulas (Batches 10, 14, and 15). Brown cells (Figure 2F) containing small iridescent  
291brown granules were also observed in about 10.83% of mussels. Their presence varied from batch to  
292batch (Table 4) and were observed in 0% to 89% of individuals, depending on the site and the  
293sampling date, and increased relative to the number of bacteria in haemolymph, reaching a proportion  
294of 6% of the circulating haemocytes in some individuals from Batch 9. Agranulocytes showed  
295aggregation capability with eosinophilic granulocytes accumulated around the aggregates. In several  
296cases, we observed “rosetting” where the central cell was a mitotic cell surrounded by agranulocytes.  
297Phagocytosed cells were sometimes observed inside granulocytes.

298The investigation on the morphology of neoplastic cells was conducted on all the DN-affected mussels  
299detected during the present study. They appeared enlarged, highly basophilic, with prominent nucleoli.  
300The scatterplot for cell diameter and NCR (Figure 3) showed that neoplastic cells were separated from  
301normal haemocytes, even if an overlap area existed with agranulocytes. Three distinct neoplastic  
302populations emerged from the analyses. The Kruskal-Wallis test showed a significant difference  
303( $p < 0.05$ ) in cell and nucleus diameters and for NCR among the three groups. These characteristics  
304corresponded only in part with the A and B types previously described (Lowe and Moore, 1978, Green  
305and Alderman, 1983; Carella et al., 2013). The most frequent cell type (approx. 40% of diseased  
306individuals), which we termed P-type (Figure 4A), had a lower NCR, a pleomorphic (usually lobed or  
307reniform) nucleus with many nucleolus-like structures and, typically, a larger cytoplasm diameter  
308when compared with the second type cells. A rarer sub-type with smaller cells and round nucleus  
309named Ps-type (approx. 4% of individuals) was also observed in a few individuals. The second most  
310prevalent cell type (approx. 32% of individuals), U-type (Figure 4B), showed a regular outline, scant  
311cytoplasm, an ovoid-shaped hyperchromatic nucleus, and one (occasionally 2) nucleolus. The third  
312cell type, S-type, occurred less frequently (approx. 17% of individuals). It was represented by smaller  
313cells, with a diameter comparable to large agranulocytes, but highly more basophilic and with at least

314one prominent nucleolus (Figure 4C). We used new terms to avoid any confusion with previous  
315descriptions: P-cells (pluri-nucleolus), and the smaller sub-type Ps-cells, U-cells (unique nucleolus),  
316and S-cell (small cells). Their morphometric characteristics are summarised in Table 3. Most  
317individuals were affected by only one of the four neoplastic cell types but in few individuals (7%),  
318both types P and U were present. Very occasionally, binucleated and multinucleated neoplastic cells  
319were observed but only in P-type affected individuals. Both cell types contained cytoplasmic vacuoles,  
320but they were particularly abundant in the P-type. Mitoses were very rare, but few multipolar atypical  
321mitoses were seen (Figure 4D).

322

323

324

325The total haemocyte count in normal mussels was estimated as a mean of  $4.4 (\pm 1.3) \times 10^6$  cells/mL but,  
326in the individuals sampled in February, haemocyte count was significantly lower, mean  $9.8 (\pm 1.2)$   
327 $\times 10^5$  cells/mL (ANOVA,  $p < 0.05$ ), in the three sampled sites, without significant inter-individual  
328variations. The proportion of granulocytes was mean  $64 (\pm 4.3) \%$ . Mitoses, easily observable at the  
329prophase and metaphase stages, were observed in most individuals and concerned both granulocytes  
330and hyalinocytes.

331Neoplastic cells were initially observed in 59 of the 1791 specimens analysed by haemocytological  
332observation (3.3%). About 59% of the 59 specimens were lightly affected, 19% moderately, and 22%  
333heavily. The neoplastic cell-type was not associated with a specific disease stage. The total haemocyte  
334count in DN-affected individuals was not significantly higher. Two other haemocytic disorders were  
335observed. Their frequencies among the batches are reported in Table 4. “Granulocytosis” consisted in  
336the significant proliferation ( $p < 0.05$ ) of eosinophilic granulocytes reaching  $>90\%$  of the circulating  
337haemocytes and a total mean haemocyte count  $>8 \times 10^6$  cells/mL. No relevant differences in typical  
338cell morphology were observed. Conversely, in some individuals we observed a predominance of  
339agranulocytes and, in some specimens, a nearly total absence of granulocytes (“granulocytopenia”)

340with a significant decrease of total mean haemocyte count ( $2.8 \times 10^6$  cells/mL). No significant  
341differences in the morphometric values were observed when compared with the agranulocytes of  
342unaffected mussels. However, aggregates were nearly absent on these smears as also observed in S-  
343type neoplasia.

344We calculated a positive correlation between the basophilic granulocytes and granulocytopenia  
345frequencies (Pearson, 0.5127,  $p=0.02$ ) but no correlation was detected between neoplasia frequencies  
346and other variables.

### 3473.3 *Histological observations*

348Neoplastic cells were easily identifiable during histological observation. They appeared as enlarged,  
349basophilic, agranular cells, with a high NCR and one or more nucleolus-like inclusions. Among the  
350402 specimens examined histologically, the result was not interpretable in three samples due to  
351degradation. Disseminated neoplasia was observed in 65 individuals. All five morphological types of  
352atypical haemocytes previously described by Carella et al. (2013) were identified. In particular, in P-  
353type neoplasia (according to haemocytological observation), we observed a high heteromorphism of  
354nuclei and numerous multinucleated cells (Figure 5). The DN advanced condition was characterised  
355by the infiltration and intense invasion of the vesicular connective tissue by enlarged, basophilic  
356neoplastic cells. Atypical cells were also present in muscle and gills, where they induced enlargement  
357of the vessels. Generally, a high mitotic rate was observed in the neoplastic compartment, usually  
358represented by atypical mitoses. In less advanced stages, the foci of neoplastic cells were present  
359around the digestive tubules, the stomach or the intestine and, less intensely, in the vesicular  
360connective tissue. No correlation between DN stage and neoplastic cell types was observed. In  
361addition, two types of inflammatory disorders were observed in about 20% of the individuals not  
362affected by DN. The first consisted of the local infiltration of connective tissue by the normal  
363inflammatory component (granulocytes and agranulocytes). The second corresponded to the  
364granulocytomas previously described by Lowe & Moore (1979). The frequency of these lesions did  
365not differ significantly among the batches and were not correlated with DN prevalence.

366

#### 3673.4. *Flow cytometry*

368A total of 331 haemolymph samples were individually analysed by flow cytometry and 277 were  
369retained for their suitable quality. In particular, part of the samples from Batches 12 and 13 were  
370excluded. These two samplings were conducted just before the spawning period, usually occurring in  
371March, and mature spermatozooids contaminated the haemolymph sample during drawing (as observed  
372in haemocytology). In mussels diagnosed as asymptomatic for DN by haemocytological observation,  
373we observed a main peak in the cytometry histogram representing the dominant cell population and  
374constituting the  $G_0/G_1$  compartment (diploid) of the normal cell cycle, and a second peak attributable  
375to the  $G_2/M$  (4N) phase (Figure 6A). However, some mussels presented more complex patterns. Twenty  
376specimens from Daoulas and few specimens from other sites (Table 5), not affected by DN in  
377haemocytology, displayed a second peak situated between the normal 2N and 4N peaks. The ploidy  
378level of this anomalous peak was  $2.92(\pm 0.29)N$ , ranging between 2.56 and 3.54N and represent  
37933.12( $\pm 10.02$ )% of the circulating cells. No haemocytic disorder, increased phagocytosis, or gamete  
380presence was observed during haemocytological and histological observations in these mussels.

381

382Eight mussels showed profiles consistent with DN. All presented a cell population with an  
383extraordinarily high ploidy, in addition to the normal  $G_0/G_1$  and  $G_2/M$  peaks (6B, 6C). The DNA  
384content of the neoplastic peak ranged from 4.18 to 9.9x the diploid DNA content, corresponding to  
3858.36 to 18.8N. The neoplastic cell population within an individual was characterised by a low  
386dispersion of the value of ploidy, as confirmed by a low coefficient of variation <6%. Only one sample  
387from St. Brieuc had two aneuploid peaks corresponding to 9.4 and 11.64N (Figure 6C). The  
388percentages of each cell population in neoplastic mussels are reported in Table 6. There was no  
389correlation between the degree of ploidy and the disease stage. On the contrary, a trendline between  
390ploidy and mean nucleus diameter of neoplastic cells, determined during haemocytological  
391observation, emerged (Figure 7).



392

393 In order to estimate the cell replication activity, we computed the fraction of cells in the S-G<sub>2</sub>/M phase.  
394 We never observed a second peak on the cytometry histogram of the DN-affected individuals that  
395 could represent the G<sub>2</sub>/M phase of the neoplastic cell population, even with the lower amplifier gain.  
396 Among the mussels with no neoplastic disorder according to haemocytological observation, we  
397 excluded those bearing the anomalous “nearly triploid” peak for the statistical analysis. The  
398 individuals from Daoulas showed a significantly higher mean percentage of cells in the S-G<sub>2</sub>/M phase  
399 compared with the other sites (Kruskal-Wallis, K= 100.57, df=5, p < 2.2e-16), representing  
400 7.45(±3.56)% vs 2.51(±1.92)%, respectively. Moreover, in 19.54% of the individuals from Daoulas,  
401 more than 10% of circulating haemocytes were in the S-G<sub>2</sub>/M phases. Only one individual from Agon  
402 exceeded slightly the 10% threshold (10.39%), considered by Elston et al. (1990) to be the upper limit  
403 to consider the mussel unaffected by genomic abnormalities. No hypodiploid peaks were observed.

#### 404 3.5. Genetic analysis

405 Genetic analysis of haemolymph DNA was conducted with 12 diagnostic SNPs between *M. trossulus*  
406 and *M. edulis*/*M. galloprovincialis* in a subsample of 114 mussels, 11 of which DN-positive with  
407 haemocytology: a sample of 100 mussels from Batch 10, 6 mussels from Batch 2 (St Brieuc,  
408 including 3 DN-positive specimens), 6 mussels from Batch 5 (Lannion, including 5 DN-positive  
409 specimens) and 2 mussels from Batch 8 and 11 (Camaret) were analysed. (Unfortunately,  
410 haemocytology and histology found only one specimen from Daoulas to be lightly affected.) The  
411 compound multilocus estimates of the *trossulus* allele fluorescence are presented in [Figure 8](#) and the  
412 single locus fluorescence values are provided in [supplementary Figure S1](#). We found six samples  
413 with evidence of amplification of *trossulus* alleles ([Figure 8](#)): two specimens diagnosed as heavily  
414 affected, three specimens diagnosed as moderately affected, and one lightly affected. Thus, 4 of the  
415 lightly affected and one of the four moderately affected specimens were negative for genetic  
416 chimerism. The four types of neoplasia were detected positive by genetic analysis.

417

#### 418 3.6. Sensitivity of the various diagnostic methods

419 In our study, 115 individuals tested DN-positive by at least one of the four diagnostic methods. Among  
420 the 572 mussels showing interpretable results with at least two diagnostic techniques, results from 12  
421 individuals were not concordant among the techniques. The individual results of the four diagnostic  
422 methods are detailed in [supplementary Table S1](#). The sensitivities of histology and haemocytological  
423 observation for DN-detection were compared in 399 mussels and results were in accordance in  
424 99.25% of cases. All heavily and moderately-affected mussels diagnosed by haemocytology were  
425 positive in histological observation, but two lightly affected individuals in haemocytology were not  
426 detected by histology. On the contrary, in a mussel diagnosed as negative by haemocytology, a few  
427 neoplastic cells were present around the stomach in histology. Flow cytometry, when compared with  
428 haemocytological observation of 277 haemolymph samples, positively diagnosed all the heavily and  
429 moderately-diseased mussels while the lightly-diseased individuals showed profiles consistent with  
430 non-diseased individuals. Nevertheless, the cytometry histogram of one individual, alternatively  
431 investigated only by haemocytology and diagnosed as non-diseased, displayed the presence of a few  
432 hyperploid cells compatible with neoplasia. The sensitivity of flow cytometry appeared significantly  
433 lower than haemocytological observation, with concordant results in 98.19% of samples. Finally,  
434 according to the genetic analysis carried out on 114 mussels, genetic chimerism was detected in all the  
435 heavily-neoplastic stages, in 75% of the moderately-affected mussels, and in 20% of the lightly  
436 neoplastic specimens. None of the non-diseased mussels was found to present this genetic anomaly.  
437 Considering the present results, haemocytological observation was chosen for the monitoring of the  
438 disease progression.

### 439 3.7. *Prevalence and monitoring of the disease progression*

440 According to haemocytological results, disseminated neoplasia was detected in all sites, except in  
441 Pénestin and Roscanvel ([Table 4](#)). Initial prevalence ranged between 0% and 25.45%, with a mean of  
442 3.29%. Neoplastic animals were found consistently in Lannion, Camaret, and Agon-Coutainville. The  
443 chi-square test revealed that the DN prevalence was higher in the groups with higher mortality  
444 ( $\chi^2(1,0.05)=53.16$ ,  $p\text{-value}=3.07\times 10^{-13}$ ). The highest prevalences were observed in Batches 1, 3, 8, and 17;  
445 however, DN was absent or prevalence was low in Batches 15 and 18, respectively, which also had

446increased mortality. Additionally, DN prevalence in Batches 4, 5, 7, and 12 was in the average range  
447but there was no increased mortality. Both species, *M. edulis* and *M. galloprovincialis*, sampled in  
448Lannion on 26<sup>th</sup> July and 1<sup>st</sup> August 2017, respectively, had comparable prevalence. Significant  
449differences ( $\chi^2$ ,  $p < 0.05$ ) were observed between both farming technique and animal age, with higher  
450prevalence in older mussels and those not exposed to tide. In all sites, we reported simultaneously the  
451presence of various neoplasia types.

452According to haemocytological observation, a total of 75 individuals of the 627 monitored were  
453diagnosed with DN during the monitoring. Twenty-two of the monitored individuals were positive  
454upon arrival and 53 developed the disease during the subsequent months. Incidence of DN between  
455two consecutive haemolymph samplings is represented in [Figure 9](#). Interestingly, in all the batches, we  
456observed an increase of incidence during the first 9 weeks followed by a decrease. In Batch 11,  
457monitored for 7 months, only two new cases appeared after the fourth month. There were fewer  
458diseased individuals at the end of the monitoring in two batches (2 and 11) that had lower initial  
459prevalences ( $\chi^2$  (4, 0.05)=14.23; p-value=0.006)).

460In the individuals that developed DN, we did not observe previous changes in the haemocytogram or  
461in cell morphology before the occurrence of the first neoplastic cells. We represented the evolution of  
462the disease in individuals acquiring the disease in [Figure 10A](#). Remission was observed in 12% of  
463diseased individuals (9/75), one previously diagnosed as high intensity stage, and concerned both P  
464and U-type neoplasia. Remission was characterized by a rapid decrease of the number of neoplastic  
465circulating cells some of which appeared picnotic or necrotic (until clearance in 3-6 weeks). The  
466remission stage was confirmed in five individuals analysed by histology; in three the remission was  
467complete and, in two, few neoplastic cells were still present in the connective tissue. No anomaly was  
468detectable by flow cytometry in the three mussels showing a total remission but, in one, the remission  
469was partial and some hyperploid cells were still present. On the contrary, genetic analyses detected the  
470presence of *trossulus* chimerism in the single individual analysed by this method and otherwise  
471presenting an apparent total remission. If we consider only individuals tested negative at the first  
472haemolymph drawing (n=53), the heavy intensity stage was usually reached 6-9 weeks after the first

473neoplastic cells appeared. However, in a few individuals, the course of the disease was more acute: in  
474six individuals the process lasted 3 weeks and in two others, after having been diagnosed negative  
475during the last haemolymph drawing, the haemocytological observations carried out at their death 10  
476and 12 days later revealed an advanced stage of the disease. Interestingly, in all individuals the same  
477neoplastic cell type was observed during all the course of the disease. Except for individuals  
478undergoing remission processes, DN-affected mussels that reached the high intensity stage usually  
479died between about 2-4 weeks later. Moreover, all the individuals diagnosed as high intensity at their  
480arrival died within the first month.

481Mortality of individuals with no DN cells occurred in all the batches, especially between the 6<sup>th</sup> and  
482the 9<sup>th</sup> weeks (Figure 10B) and some batches experienced high losses (48% for Batch 2). In most of  
483these cases, moribund individuals analysed by histology showed haemocytic infiltration and  
484granulomas associated with bacterial invasion.

#### 4854. Discussion

486We studied the haemolymph of 1791 mussels *Mytilus* sp. using cytological analysis. Haemocytes  
487constitute the cellular component of haemolymph and their morphological classification was a  
488prerequisite to further exploring the DN development. However, controversy over their  
489characterisation remains. As in previous studies (Rasmussen et al., 1985; Carballal et al., 1997), we  
490classified the circulating haemocytes of both *M. edulis* and *M. galloprovincialis* specimens in two  
491morphologically distinct cell types: granulocytes and agranulocytes, depending on the presence of  
492cytoplasmic granules. The distinction between two types of granulocytes suggested by Pipe (1990),  
493some with small or large granules, was not univocal in our samples because granule dimension varied  
494in a single cell. Carballal et al. (1997) observed bi/multi-nucleated granulocytes in a mussel parasitized  
495by trematodes. The presence of basophilic granulocytes appeared to be very limited; they were  
496observed in less than 2% of individuals and represented only few cells, but were reported from nearly  
497all sampling sites. Le Foll et al. (2010) reported a higher proportion of basophilic granulocytes in  
498haemolymph (20-25%). We did not observe the granulocytes containing both types of granules  
499(eosinophilic and basophilic) described by Carballal et al. (1997). These differences could be

500 attributed to different physiological or environmental conditions among the studies. We observed the  
501 small and large agranulocytes that Moore and Lowe (1977) suggested considering as two sub-  
502 populations. Brown cells were observed in the haemolymph of some individuals, corresponding  
503 morphologically to the brown cells described in mussel tissues by Moore and Lowe (1977) and  
504 Carballal et al. (1997). We observed that their presence in the haemolymph was linked to the invasion  
505 of bacteria and the number of cells was particularly high in the batch from Lannion, sampled on 20<sup>th</sup>  
506 July during a mortality event. Granular haemocytes were the major constituent of the circulating cell  
507 populations. The haemocytograms from mussels of all origins showed a similar pattern with no  
508 significant differences except for the two batches sampled in February, both with a lower total  
509 haemocyte count. A seasonal evolution in total haemocyte count was previously observed in bivalves,  
510 linked to water temperature variations or gonadal development (Soudant et al., 2004; da Silva et al.,  
511 2008; Flye-Sainte-Marie et al., 2009; Perrigault et al., 2011) but also to the presence of pathogens  
512 (Parisi et al., 2008; Ciacci et al., 2009) or pollution (Auffret et al., 2006). Granulocytosis and  
513 granulocytopenia were also observed in approximately 4% and 8% of individuals, respectively, but  
514 further studies are needed to understand their causes and the mechanisms leading to these conditions.  
515 In fact, haemocyte populations might vary greatly with environmental and physiological conditions or  
516 in presence of pollutants and pathogens (Pipe and Coles, 1995; Soudant et al., 2004; da Silva et al.,  
517 2008; Donaghy and Volety, 2011). By quantifying the mitotic activity of haemolymph cells, Mayrand  
518 et al. (2005) showed that blue mussels exposed to a stressor were able to control the number of  
519 circulating haemocytes by adjusting the mitotic activity of haemocytes. Contrary to reports by Moore  
520 and Lowe (1977) in *M. edulis* and Matozzo et al. (2008) in *Ruditapes decussatus*, we did not observe  
521 mitotic figures in the small agranular cells but mitoses were observed in granulocytes and, more rarely,  
522 in the large agranular cells, questioning the function as stem cells attributed to small agranulocytes.  
523 The haematopoiesis in bivalves is still a debated topic, principally due to the lack of specific markers.  
524 There are several theories on bivalve haemocyte renewal and maturation (see review Pila et al., 2016).  
525 A definitive haematopoietic site has yet to be identified.

526 Our first goal was to identify the best method to diagnose and follow the evolution of DN. We showed  
527 that drawing haemolymph samples was non-destructive and individuals can be repeatedly sampled  
528 without affecting survival, as also demonstrated in *Mya arenaria* (Brown et al., 1978; Cooper et al.,  
529 1982). This finding is relevant for future studies on mussel haemolymph. We showed that the  
530 sensitivity of haemocytological observation appears to be appropriate for DN diagnosis, comparable to  
531 that of histology in early stages of the disease. The relative accuracy of these two approaches is most  
532 likely a function of the relative amount of tissue that is processed and examined. Haemocytological  
533 observation detected one neoplastic cell among about 150,000 fixed on the slide. Flow cytometry and  
534 genetics appeared to be reasonable diagnostic methods but might not be sufficiently sensitive in early  
535 stages of the disease. However, genetic analysis is the only method that can diagnose neoplasia as a  
536 *trossulus* transmissible cancer. Although the fluorescence signal is likely too low for lightly infected  
537 specimens, it remains possible that some neoplasms were not the *trossulus* transmissible cancer.

538 Neoplastic cells were easily identifiable by haemocytology and histology because of their peculiar  
539 morphology compared to normal haemocytes. They were characterised by a larger diameter, higher  
540 nuclear-cytoplasm ratio, prominent nucleoli and marked basophilia. Histological observation revealed  
541 that, in tissues, the neoplastic cell population within a specimen was heteromorphic, confirming the  
542 observations of Carella et al. (2013). In contrast, the population circulating in the haemolymph  
543 appeared to be relatively homogeneous. Among mussels with DN, four types of anomalous cells were  
544 observed in the haemolymph (P-type, Ps-type, U-type, and S-type) but usually only one type was  
545 detected in a single individual. These cell-types clearly differed in size, NCR, nucleus shape, number  
546 of nucleoli, and basophilia. The P-type was the most frequent form of neoplasia. Previous studies,  
547 conducted on other bivalve species, described only two neoplastic circulating cell-types, type A and  
548 type B (Farley and Sparks, 1970; Lowe and Moore, 1978; Green and Alderman, 1983) that correspond  
549 only partially to P-type and U-type of our study. We did not find a link between cell type and disease  
550 stage, thus it is unlikely that the various cell types correspond to a continuum of differentiation. Cells  
551 undergoing mitosis and plurinucleated cells were common in tissues, as reported in previous studies  
552 (Mix et al., 1979; Carella et al., 2013) but were rare among the circulating cells.

553Neoplastic cells were also easily distinguishable by flow cytometry due to their high DNA content,  
554ranging between about 8 and 18 times the normal haploid DNA content, depending on the individual.  
555Clearly, a relation between the nucleus size (measured by haemocytological observation) and ploidy  
556was established allowing a predictive estimation of the DNA content. The low coefficient of variation  
557in fluorescence values among the neoplastic cells of a single mussel showed that the pattern of DNA  
558content of this population within the host was uniform with a comparable ploidy in all the circulating  
559anomalous cells. Only one individual presented simultaneously two neoplastic cell populations,  
560distinct for their ploidy but not enough to be distinguishable morphologically. The high DNA content  
561of neoplastic cells in mussels was mentioned in previous reports, even when different degrees of  
562ploidy were reported. Interestingly, we observed the absence of correlation between the disease stage  
563and ploidy value. This conclusion contrasted with the results of Benabdelmouna et al. (2018) in  
564mussels and Le Grand et al. (2010) in cockles, who suggested an evolutive continuum of ploidies  
565during disease development. However, these two studies did not use genetic analysis, which might  
566have determined if they were describing the transmissible neoplasia we studied. Our results are more  
567consistent with the observations of neoplasia in *M. trossulus* by Moore et al. (1991) and Elston et al.  
568(1990), which were likely transmissible neoplasias (Metzger, 2016). These authors showed that  
569neoplasia exists in two forms in this host, pentaploid and tetraploid, representing distinct pathogenetic  
570processes rather than sequential stages of a single pathogenesis. In our study concerning *M. edulis*  
571hosts, the definition of disease forms according to ploidy was not so conclusive as a high inter-  
572individual variability existed. Unfortunately, all previous flow cytometry analyses carried out on  
573mussel DN, except the study of Benabdelmouna et al. (2018), concerned *M. trossulus* hosts and  
574therefore the descriptions of the ploidy patterns are limited for *M. edulis* and *M. galloprovincialis*. In  
575our samples, the number of circulating neoplastic cells in the S/G2M phases was very low, to such an  
576extent that no corresponding peak was graphically distinguishable, whereas Elston et al. (1990) and  
577Moore et al (1991) in *M. trossulus*, described a cell population in the S/G2M phases. Our observations  
578in flow cytometry confirmed the low mitotic rate detected by haemocytological observation.

579The genetic analysis shows that DN of the French mussels we studied is a *trossulus* transmissible  
580cancer, as proposed by Riquet et al. (2017). *Trossulus* alleles in haemolymph DNA of *M. edulis* hosts  
581were amplified regardless of the type of neoplasia. Although the four DN types might correspond to  
582different lineages of transmissible cancer, our results with 12 diagnostic genetic markers show they all  
583share a *M. trossulus* origin. Further investigations will be required to determine if the different types  
584are sublineages of the same emergence or independent emergences. Of interesting note, no *trossulus*  
585individuals have been identified previously in France. Questions remain regarding how and when this  
586neoplasm colonized *M. edulis* mussels in France from its *M. trossulus* population of origin: (i)  
587naturally by stepping stone propagation from *M. trossulus* populations, and it has been missed until  
588now or (ii) by recent anthropogenic induced migration.

589Disseminated neoplasia in mussels was widespread among the sampling sites and was found to affect  
590both *M. edulis* and *M. galloprovincialis*. No geographic distribution of the various neoplasia types  
591emerged. Interestingly, older individuals and stocks not subjected to tide (farmed in suspension) were  
592most affected, suggesting that they are promoting factors. It is not clear if it is time of exposure that  
593increases the probability of infection or if adult stages are more vulnerable and if the culture technique  
594in suspension is a particular source of physiological stress for these species. Seasonality in prevalence  
595was difficult to assess because it was not possible to sample the same batch over a longer time period  
596due to the short farming cycle of mussels.

597We detected disseminated neoplasia in locations affected by higher mortality as well as in mortality-  
598free sites (Bernard et al., 2017, 2018; REPAMO [https://wwz.ifremer.fr/sante\\_mollusques](https://wwz.ifremer.fr/sante_mollusques)), and DN  
599was not detected in the Daoulas site in May 2018 during an event of high mortality. This site regularly  
600has higher than normal mortality but DN was rare (only one positive of the 300 mussels tested).  
601However, 23% of the individuals from this batch showed a supplementary population of nearly triploid  
602cells during flow cytometry analysis while no anomalies were observed in haemocytological  
603observation. A similar condition with a second slightly hyperdiploid peak was observed in *M.*  
604*trossulus* by Elston et al. (1990), who suggested it to be a distinct normal cell population. In the same  
605Daoulas batch, other 20% of individuals presented with more than 10% of circulating haemocytes in



606the S-G2/M phases. This kind of genomic abnormality, the origin of which is unknown but may be  
607promoted by contaminants, was linked to mortalities by Benabdelmouna and Ledu (2016).  
608Nevertheless, the prevalence of DN was above the mean value of 3.29% in four of the six batches  
609regularly affected by high mortality, but was also far lower than the mortality rate recorded. According  
610to these observations, it was not possible to assign the cause of mortality to DN alone.

611Our efforts to anticipate the diagnosis of the disease by identifying early indicators were not  
612conclusive. No correlation between neoplasia and other variables (haemocytogram values,  
613inflammatory lesions such as granulocytomas and haemocytic infiltration, and early genomic  
614abnormalities) was demonstrated. Presently, the disease seems to be diagnosable only when  
615characteristic neoplastic cells appear. However, with the evidence of the genetic peculiarity of  
616neoplastic cells, a molecular test that is rapid, sensitive and specific for DN is likely to be developed  
617in the near future. According to the results of our monitoring, carried out by haemocytological  
618observation of mussels maintained under controlled and stable conditions, disease incidence was not  
619constant over time but followed the same trend in all the batches, reaching a maximum after 2 months  
620and then decreasing. Various interpretations are possible: i) the infection occurred in the natural  
621environment before the collection and was not transmitted under the laboratory conditions, even if at  
622least one DN-affected mussel was always present in each tank; ii) the sensitivity to DN varies among  
623individuals and some of them, the survivors, are resistant; iii) physiological conditions are  
624determinants for the development of the disease. Assuming the first hypothesis is supported, it would  
625mean that we were not able to detect the first stage of infection for months and suggests the existence  
626of a quiescent state. Disease progression in affected mussels appeared usually rather slow to reach the  
627complete replacement of normal haemocytes by neoplastic cells, followed by death about 1 month  
628later. Disease progression is, however, poorly generalisable. In fact, in few cases, we observed a rapid  
629and total invasion of haemolymph by neoplastic cells in less than 2 weeks, probably promoted by  
630stress factors or due to a more aggressive DN form. Unfortunately, because our monitored batches  
631suffered mortality not attributable to DN, we were not able to evaluate consistently whether  
632differences exist between the various forms of neoplasia. Finally, remission of DN was observed and

633this proved to be quite frequent (12%), regardless of the neoplasia type. A higher remission rate (20%)  
634was observed in *M. trossulus* (Elston et al., 1988), confirming that it is common in the course of DN  
635infection. The rapid decrease of the number of circulating anomalous cells during remission was also  
636observed in tissues. Interestingly, the DNA of neoplastic cells was still detectable after complete  
637remission by genetic analysis. Two assumptions can be made i) *trossulus* DNA resulting from the  
638destruction of neoplastic cells is still present, free or within phagosomes, ii) *trossulus* cells are still  
639present in the haemolymph but are undistinguishable from host haemocytes at this stage.

640Presently, it is not possible to conclude that mussel mortality events in northern France are attributable  
641to DN. We observed neoplasia in batches not affected by mortality and, on the contrary, the absence of  
642neoplasia in batches suffering these events. Prevalence was higher than the mean prevalence in four of  
643the six batches sampled during mortality events; however, during the monitoring in laboratory, we  
644observed a contemporaneity between an increase in mortality in DN-unaffected individuals and an  
645increase in DN incidence. This may indicate that mussels are developing a particular sensitivity to  
646pathogens and the promotion of DN development. DN is particularly interesting due to its peculiarity  
647among neoplasms of being directly transmissible from individual to individual and across species  
648(here from *M. trossulus* to *M. edulis*). The infection route remains to be determined.

#### 6495. **Conclusions**

650In the present study, we obtained information on the circulating cells of the blue mussel and on  
651disseminated neoplasia. We observed that DN is a widespread disease in mussels in northern France,  
652although prevalence is low. Furthermore, various types of DN coexist in the same site. Our genetic  
653studies showed that *trossulus* DNA amplification was associated with most of the DN cases in *Mytilus*  
654*edulis*, regardless the type. Interestingly, we observed a high diversity of forms and high mitotic rate of  
655neoplastic cells located in the vesicular connective tissue and not in haemolymph, which raised the  
656question of the proliferation site of these cells.

657Some observations raised new questions:

658 We described the normal cellular composition of the haemolymph of blue mussels and detected that  
659 some variations occur. Further studies are needed to understand the origin, the consequences, and the  
660 function of these alterations.

661 We detected *trossulus* DNA in the haemolymph of mussels with DN, consistent with a transmissible  
662 cancer; however, the route of infection and the invasive mechanism are still unknown.

663 We demonstrated that haemocytological examination is a good method to diagnose DN and monitor  
664 the disease; however, it is not sufficient to understand the details of DN pathogenesis, and a  
665 comparison between the neoplastic cells in tissues and those circulating in the haemolymph is  
666 necessary, along with the development of specific cellular markers.

667 We detected genomic anomalies, different from DN, that may be linked with host mortality and was  
668 already described in previous studies associated with mortality events; however, the cause of these  
669 anomalies and the real link with mortalities is still unclear;

670 We produced data suggesting that DN is not alone responsible for the mortality outbreaks in farmed  
671 mussel stocks in northern France; however, the real impact of DN on an entire cycle of production  
672 should be evaluated. Because of the economic relevance of mussel farming in Europe, all these topics  
673 should be the subject of further studies, aiming to contribute to reduction of mortality events.

674

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681

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