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

Burioli E.A.V1*, Trancart S.1, Simon A.2, Bernard I.3, Charles M.1, Oden E.1, Bierne N.2, Houssin M.1,4

*Corresponding author: erikastrid.burioli@gmail.com, LABÉO, 1 Route de Rosel, 14280 St Contest, France

Abstract

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

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

### 1. Introduction

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

Various aetiological origins have been suggested. Exposure to pollutants and sublethal levels of biotoxins were suspected to be involved in DN development (Lowe and Moore, 1978; Reinisch et al., 1984; Landsberg, 1996) but was not confirmed in subsequent studies (Green and Alderman, 1983). On the other hand, the transmission of the disease between mollusc individuals including mussels, through
cohabitation or haemolymph injection, has been proven (Elston et al., 1988; Twomey and Mulcahy, 1988; Kent et al., 1991; House, 1997; Weinberg et al., 1997), indicating that an infective agent, or the neoplastic cells themselves, could be involved. DN transmission has been effected by injection of neoplastic whole cell preparations, but when cell-free 0.45 μm filtered preparations were used, conclusions by different researchers on the success of the disease transmission were not unanimous (McLaughlin et al., 1992; House et al., 1998; Collins and Mulcahy, 2003).

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

Mussel farming is of great economic importance in several European countries. French production is represented by two species, *M. edulis* and *M. galloprovincialis*, which are able to hybridise in several areas where they coexist (Bierne et al., 2003). Production in France amounts to around 75,000 tons each year (FAO, 2016). However, since 2014, mass mortality events (90-100%) have occurred in France, beginning in the Pertuis Charentais (Nouvelle-Aquitaine, France) (Béchemin et al., 2015) and, since 2016, in other farming areas in Brittany (France) (Bernard and Allain, 2017, Bernard et al., 2017; 2018). The causes of these mortality events are still unresolved. Some studies hypothesized the involvement of *Vibrio* species (Ben Cheikh et al., 2016, 2017; Oden et al., 2016), probably coupled with stressful environmental conditions (Polsenaere et al., 2017). Benabdelmoua and Leduc (2016)
associated abnormal outbreaks of mussel mass mortality with poor cytogenetic quality, particularly in terms of ploidy variations in circulating cells. In a second study, Benabdelmouna et al. (2018) linked these genomic abnormalities to ongoing neoplastic processes. DN was also suspected to be involved in these events after the finding of a high prevalence of DN in a *M. edulis* collection from Lannion (France) affected by mortality in October 2016 (Burioli et al., 2017). Massive mortalities of economically important bivalve stocks have occasionally been linked to high prevalence of DN in other parts of the world (Frierman and Andrews, 1976; Elston et al., 1992; McGladdery et al., 2001; Villalba, et al., 2001).

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

2. Materials and Methods

2.1. Sampling

Eighteen batches of mussels were collected during sampling campaigns conducted between July 2017 and June 2018. A total of 2,188 mussels were collected from six farming sites and a wild population (Table 1 and Figure 1). We also reported the origin areas where mussel spat was collected from the wild in the prior year before being moved to their final growing sites. At time of sampling, individuals from 12 batches were more than 1 year in age, while the others were aged approximately 1 year or less (the age of wild individuals is unknown). Sampled mussels were farmed on the intertidal zone (Agon-Coutainville, St Brieuc, Daoulas, and Pénestin) or in suspension in deep waters (Lannion and Camaret). Wild mussels (Roscanvel) were collected on the intertidal zone. Sixteen batches were
previously identified as mainly *M. edulis* and two as mainly *M. galloprovincialis* according to morphological characters and by genotyping the repetitive region of foot protein 1 in 10 individuals per batch according to Inoue et al. (1995).

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

### 2.2. Evaluation of the various diagnostic methods

Because we wanted to maintain mussels alive with the aim to study the incidence of DN and the progression of the disease, we tested the non-invasiveness of regular haemolymph drawing by comparing the mortality of sampled and naïve individuals. Mortality was monitored daily for 2 months in four mussel batches (N=791) (see Table 1). Cumulative mortality rates were compared for individuals from which 100 µL of haemolymph was drawn every 3 weeks and those not subjected to haemolymph drawing (naïve) (see Table 2). Once the mussel batches arrived at the LABÉO laboratory, the groups used for this trial were subdivided and held in 10-L tanks, a maximum of 100 individuals per tank, in natural sea water maintained at 15°C, under oxygenation conditions. Complete water changes were done on alternate days.
Haemocytological observation was conducted on all batches on their arrival at the laboratory. The sensitivities of haemocytological observation and flow cytometry were then compared and accompanied by histological observation and genetic analysis in some batches (see Table 1). A total of 331 individuals underwent haemolymph drawing to allow both cytological observation and flow cytometry. We used a neoplastic individual from Batch 2 to implement the flow cytometry protocol. All individuals from the Batches 10, 12, and 13 (N=241) were simultaneously subjected to both analyses on their arrival; flow cytometry was not immediately carried out for Batch 11, but on survivors in February alongside haemocytological observation; for Batches 2 and 5, flow cytometry was carried out on randomly sampled individuals. Histological observation is an invasive method. Thus, when the batch was kept in tank for a long period of time in order to study the DN incidence and disease progression in affected mussels (Batches 2, 4, 5, and 8), histology was carried out on each moribund individual (along with haemocytological observation) and on half of the survivors at the end of the monitoring period (402 individuals in total). Genetic analysis was conducted on 114 individuals also subjected to haemocytological observation, flow cytometry, and histology. The various protocols used are reported below.

2.3. Haemocytological examination

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

The entire area of the cell spot on the cytoslide was scanned in all samples to search for neoplastic cells, which are characterised by an enlarged diameter, elevated nucleus/cell diameter ratio (NCR), and high basophilia. Four developmental stages of DN were differentiated according to the proportion of neoplastic cells in haemolymph: “non-diseased” when no neoplastic cells were observed, “low
intensity” (less than 20% of neoplastic cells), “moderate intensity” (20% to 75%), and “high intensity” (more than 75%).

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

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

\[
\text{No. haemocytes} / \text{mL haemolymph} = \frac{\sum \text{haemocytes counted} X \text{area of spot on cytoslide (mm}^2) X 1}{\text{No. fields counted} X \text{area of microscope field (mm}^2) X \text{volume of haemolymph drawn (mL)}}
\]

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

2.4. Flow cytometry analysis
When flow cytometry analysis was conducted on mussel individuals at the same time as cytological analysis, a supplementary volume of 100 µL haemolymph was drawn and placed in a 1.5-mL microtube; 500 µL of ethanol 100% was added to fix the cells, prevent aggregation, and increase cell permeability. This suspension was stored at -20° C until the next steps of the process. First, each aliquot of haemolymph was centrifuged at 600x g for 5 min and the pellet was resuspended in 500 µL sterile artificial seawater (1 L of reverse osmosis purified water, 23 g of sodium chloride, 1.49 g of potassium chloride, 0.3 g of calcium chloride, and 1.23 g of magnesium sulfate heptahydrate) for 15 min at room temperature. A second centrifugation at 600 x g for 5 min was carried out and the pellet was treated in 500 µL of propidium iodide (PI) solution: PI (Life technologies) 0.05 mg/mL; RNase A (Life technologies) 0.02 mg/mL; PBS (Eurobio 1X). Cells were stored for 45 min in the dark at room temperature. Haemocytes were then analysed by flow cytometry using a Cytoflex (Beckman Coulter) equipped with a laser providing 50 mW at wavelength 488 nm. For each individual, 10,000 cells were analysed at a medium flow rate (30 µL/min) to collect multiparametric data. The measurement of DNA contents in haemolymph cells was based on the properties of PI, which intercalate into nucleic acid molecules. When PI binds to nucleic acids its fluorescence intensity is proportional to the DNA cell content, thus allowing the study of cell ploidy (Krishan, 1975). Because some cell populations had extraordinarily high ploidy levels, each sample was run twice with two different gain settings. In order to remove doublets from single cells, the IPBLUE-height versus IPBLUE-area dot-plot was used and a region comprising single cells was drawn on this representation (adapted from da Silva et al., 2005), doublets being excluded on the right of the selected area. The cells included in this region were then plotted on a histogram in linear scale, used to calculate the percentage of cell populations according to their DNA content. In particular, the percentages of cells in the normal diploid G₀/G₁ and S/G₂/M phases were estimated according to Delaporte et al. (2008) and Benabdelmouna and Ledu (2016).

2.5. Histological examination

A standard section of the mussel body, ~5 mm thick, containing the digestive gland, gut, mantle, gills, and foot was fixed for 48 hrs in Davidson’s fixative. Fixed tissues were then processed for routine histological examination and 4-µm thick sections were stained with Harris’s haematoxylin and eosin.
Histological sections were examined under a light microscope for the presence of abnormal cells related to neoplasia.

### 2.6. Genetics

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

### 2.7. Determination of DN prevalence, incidence and progression

The occurrence of DN in mussels was described in terms of prevalence, defined as the number of mussels presenting anomalous cells in 100 individuals investigated by cytological examination. We determined the initial DN prevalence in each of the 18 batches and the developmental stage of the disease in affected animals. The incidence of the disease, defined as the number of new cases observed in survivors between two consecutive haemolymph drawings expressed in percentage, and its progression in affected individuals.
were evaluated in five batches (2, 4, 5, 8, and 11). Individuals were maintained in six different 10-L tanks (two tanks were used for Batch 11), in natural sea water maintained at around 15° C, under oxygenation conditions. Complete water changes were done on alternate days. Haemolymph drawings and haemocytological observations were carried out on all specimens, each month, for 4 to 7 months according to the batch (Table 1). In individuals that developed DN during the monitoring, a retrospective observation of haemocytological slides was carried out in search of haemocytogram modifications or changes in cell morphology that could represent a pre-neoplastic stage.

2.8. Statistical analysis

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

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

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

3.1. Haemolymph drawing is non-invasive

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

3.2. Haemocytological observations

In DN-unaffected individuals, cytological examination confirmed the presence of two main cellular types, eosinophilic granulocytes (Figure 2A) and agranulocytes (Figure 2B and 2C). Granulocytes were characterised by the presence of abundant brick-red granules in a beige cytoplasm, which varied in number and size. The nuclei were typically spherical and occasionally ovoid and eccentric. A small number of granulocytes were observed to contain two or more nuclei (Figure 2D). Lucent vesicles, consistent with phagosomes, were sometimes observed in these cells. The cytoplasm of agranulocytes appeared bluish and no granules were present. However, this population appeared more heterogeneous, in terms of size, nucleus/cell diameter ratio (NCR), basophilia, and nucleus shape. We observed highly basophilic small cells (Figure 2B) with a very high NCR and larger cells, characterised by a light-blue cytoplasm and a lower NCR with some cells showing a U-shaped nucleus (Figure 2C). In some individuals, very pale red or blue vesicle coloration was observed in a few cells. The results of the morphometric analysis carried out on cell and nucleus diameters and the NCR values are reported in Table 3. Data collected from granulocytes showed a normal distribution while those from agranulocytes did not. According to both parametric (ANOVA) and nonparametric (Kruskal-Wallis) tests, no significant differences were observed among inter-individual and inter-site measurements. From the first haemolymph drawing, we observed the presence of a few basophilic...
granulocytes (Figure 2E), smaller than eosinophilic granulocytes, characterised by numerous small blue granules in the cytoplasm in about 1.90% mussels (Table 4). When present, these cells represented less than 1% of the total haemocyte population. They were consistently present in the batches from Daoulas (Batches 10, 14, and 15). Brown cells (Figure 2F) containing small iridescent brown granules were also observed in about 10.83% of mussels. Their presence varied from batch to batch (Table 4) and were observed in 0% to 89% of individuals, depending on the site and the sampling date, and increased relative to the number of bacteria in haemolymph, reaching a proportion of 6% of the circulating haemocytes in some individuals from Batch 9. Agranulocytes showed aggregation capability with eosinophilic granulocytes accumulated around the aggregates. In several cases, we observed “rosetting” where the central cell was a mitotic cell surrounded by agranulocytes.

Phagocytosed cells were sometimes observed inside granulocytes.

The investigation on the morphology of neoplastic cells was conducted on all the DN-affected mussels detected during the present study. They appeared enlarged, highly basophilic, with prominent nucleoli. The scatterplot for cell diameter and NCR (Figure 3) showed that neoplastic cells were separated from normal haemocytes, even if an overlap area existed with agranulocytes. Three distinct neoplastic populations emerged from the analyses. The Kruskal-Wallis test showed a significant difference in cell and nucleus diameters and for NCR among the three groups. These characteristics corresponded only in part with the A and B types previously described (Lowe and Moore, 1978, Green and Alderman, 1983; Carella et al., 2013). The most frequent cell type (approx. 40% of diseased individuals), which we termed P-type (Figure 4A), had a lower NCR, a pleomorphic (usually lobed or reniform) nucleus with many nucleolus-like structures and, typically, a larger cytoplasm diameter when compared with the second type cells. A rarer sub-type with smaller cells and round nucleus named Ps-type (approx. 4% of individuals) was also observed in a few individuals. The second most prevalent cell type (approx. 32% of individuals), U-type (Figure 4B), showed a regular outline, scant cytoplasm, an ovoid-shaped hyperchromatic nucleus, and one (occasionally 2) nucleolus. The third cell type, S-type, occurred less frequently (approx. 17% of individuals). It was represented by smaller cells, with a diameter comparable to large agranulocytes, but highly more basophilic and with at least
one prominent nucleolus (Figure 4C). We used new terms to avoid any confusion with previous
descriptions: P-cells (pluri-nucleolus), and the smaller sub-type Ps-cells, U-cells (unique nucleolus),
and S-cell (small cells). Their morphometric characteristics are summarised in Table 3. Most
individuals were affected by only one of the four neoplastic cell types but in few individuals (7%),
both types P and U were present. Very occasionally, binucleated and multinucleated neoplastic cells
were observed but only in P-type affected individuals. Both cell types contained cytoplasmic vacuoles,
but they were particularly abundant in the P-type. Mitoses were very rare, but few multipolar atypical
mitoses were seen (Figure 4D).

The total haemocyte count in normal mussels was estimated as a mean of 4.4 (±1.3) x10^6 cells/mL but,
in the individuals sampled in February, haemocyte count was significantly lower, mean 9.8 (± 1.2)
x10^5 cells/mL (ANOVA, p<0.05), in the three sampled sites, without significant inter-individual
variations. The proportion of granulocytes was mean 64 (± 4.3) %. Mitoses, easily observable at the
prophase and metaphase stages, were observed in most individuals and concerned both granulocytes
and hyalinocytes.

Neoplastic cells were initially observed in 59 of the 1791 specimens analysed by haemocytological
observation (3.3%). About 59% of the 59 specimens were lightly affected, 19% moderately, and 22%
heavily. The neoplastic cell-type was not associated with a specific disease stage. The total haemocyte
count in DN-affected individuals was not significantly higher. Two other haemocytic disorders were
observed. Their frequencies among the batches are reported in Table 4. “Granulocytosis” consisted in
the significant proliferation (p<0.05) of eosinophilic granulocytes reaching >90% of the circulating
haemocytes and a total mean haemocyte count >8 x 10^6 cells/mL. No relevant differences in typical
cell morphology were observed. Conversely, in some individuals we observed a predominance of
agranulocytes and, in some specimens, a nearly total absence of granulocytes (“granulocytopenia”)
with a significant decrease of total mean haemocyte count (2.8 x 10^6 cells/mL). No significant differences in the morphometric values were observed when compared with the agranulocytes of unaffected mussels. However, aggregates were nearly absent on these smears as also observed in S-type neoplasia.

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

### Histological observations

Neoplastic cells were easily identifiable during histological observation. They appeared as enlarged, basophilic, agranular cells, with a high NCR and one or more nucleolus-like inclusions. Among the 402 specimens examined histologically, the result was not interpretable in three samples due to degradation. Disseminated neoplasia was observed in 65 individuals. All five morphological types of atypical haemocytes previously described by Carella et al. (2013) were identified. In particular, in P-type neoplasia (according to haemocytological observation), we observed a high heteromorphism of nuclei and numerous multinucleated cells (Figure 5). The DN advanced condition was characterised by the infiltration and intense invasion of the vesicular connective tissue by enlarged, basophilic neoplastic cells. Atypical cells were also present in muscle and gills, where they induced enlargement of the vessels. Generally, a high mitotic rate was observed in the neoplastic compartment, usually represented by atypical mitoses. In less advanced stages, the foci of neoplastic cells were present around the digestive tubules, the stomach or the intestine and, less intensely, in the vesicular connective tissue. No correlation between DN stage and neoplastic cell types was observed. In addition, two types of inflammatory disorders were observed in about 20% of the individuals not affected by DN. The first consisted of the local infiltration of connective tissue by the normal inflammatory component (granulocytes and agranulocytes). The second corresponded to the granulocytomas previously described by Lowe & Moore (1979). The frequency of these lesions did not differ significantly among the batches and were not correlated with DN prevalence.
3.4. Flow cytometry

A total of 331 haemolymph samples were individually analysed by flow cytometry and 277 were retained for their suitable quality. In particular, part of the samples from Batches 12 and 13 were excluded. These two samplings were conducted just before the spawning period, usually occurring in March, and mature spermatozoids contaminated the haemolymph sample during drawing (as observed in haemocytology). In mussels diagnosed as asymptomatic for DN by haemocytological observation, we observed a main peak in the cytometry histogram representing the dominant cell population and constituting the G₀/G₁ compartment (diploid) of the normal cell cycle, and a second peak attributable to the G₂M (4N) phase (Figure 6A). However, some mussels presented more complex patterns. Twenty specimens from Daoulas and few specimens from other sites (Table 5), not affected by DN in haemocytology, displayed a second peak situated between the normal 2N and 4N peaks. The ploidy level of this anomalous peak was 2.92(±0.29)N, ranging between 2.56 and 3.54N and represent 33.12(±10.02)% of the circulating cells. No haemocytic disorder, increased phagocytosis, or gamete presence was observed during haemocytological and histological observations in these mussels.

Eight mussels showed profiles consistent with DN. All presented a cell population with an extraordinarily high ploidy, in addition to the normal G₀/G₁ and G₂/M peaks (6B, 6C). The DNA content of the neoplastic peak ranged from 4.18 to 9.9x the diploid DNA content, corresponding to 8.36 to 18.8N. The neoplastic cell population within an individual was characterised by a low dispersion of the value of ploidy, as confirmed by a low coefficient of variation <6%. Only one sample from St. Brieuc had two aneuploid peaks corresponding to 9.4 and 11.64N (Figure 6C). The percentages of each cell population in neoplastic mussels are reported in Table 6. There was no correlation between the degree of ploidy and the disease stage. On the contrary, a trendline between ploidy and mean nucleus diameter of neoplastic cells, determined during haemocytological observation, emerged (Figure 7).
In order to estimate the cell replication activity, we computed the fraction of cells in the S-G2/M phase. We never observed a second peak on the cytometry histogram of the DN-affected individuals that could represent the G2/M phase of the neoplastic cell population, even with the lower amplifier gain. Among the mussels with no neoplastic disorder according to haemocytological observation, we excluded those bearing the anomalous “nearly triploid” peak for the statistical analysis. The individuals from Daoulas showed a significantly higher mean percentage of cells in the S-G2/M phase compared with the other sites (Kruskal-Wallis, $K= 100.57$, df=5, $p < 2.2e-16$), representing $7.45(±3.56)\%$ vs $2.51(±1.92)\%$, respectively. Moreover, in 19.54% of the individuals from Daoulas, more than 10% of circulating haemocytes were in the S-G2/M phases. Only one individual from Agon exceeded slightly the 10% threshold (10.39%), considered by Elston et al. (1990) to be the upper limit to consider the mussel unaffected by genomic abnormalities. No hypodiploid peaks were observed.

3.5. Genetic analysis

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

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

3.7. Prevalence and monitoring of the disease progression

According to haemocytological results, disseminated neoplasia was detected in all sites, except in Pénestin and Roscanvel (Table 4). Initial prevalence ranged between 0% and 25.45%, with a mean of 3.29%. Neoplastic animals were found consistently in Lannion, Camaret, and Agon-Coutainville. The chi-square test revealed that the DN prevalence was higher in the groups with higher mortality ($\chi^2_{(1,0.05)}=53.16$, p-value=$3.07\times10^{-13}$). The highest prevalences were observed in Batches 1, 3, 8, and 17; however, DN was absent or prevalence was low in Batches 15 and 18, respectively, which also had...
increased mortality. Additionally, DN prevalence in Batches 4, 5, 7, and 12 was in the average range but there was no increased mortality. Both species, *M. edulis* and *M. galloprovincialis*, sampled in Lannion on 26th July and 1st August 2017, respectively, had comparable prevalence. Significant differences ($\chi^2$, p<0.05) were observed between both farming technique and animal age, with higher prevalence in older mussels and those not exposed to tide. In all sites, we reported simultaneously the presence of various neoplasia types.

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

In the individuals that developed DN, we did not observe previous changes in the haemocytogram or in cell morphology before the occurrence of the first neoplastic cells. We represented the evolution of the disease in individuals acquiring the disease in Figure 10A. Remission was observed in 12% of diseased individuals (9/75), one previously diagnosed as high intensity stage, and concerned both P and U-type neoplasia. Remission was characterized by a rapid decrease of the number of neoplastic circulating cells some of which appeared picnotic or necrotic (until clearance in 3-6 weeks). The remission stage was confirmed in five individuals analysed by histology; in three the remission was complete and, in two, few neoplastic cells were still present in the connective tissue. No anomaly was detectable by flow cytometry in the three mussels showing a total remission but, in one, the remission was partial and some hyperploid cells were still present. On the contrary, genetic analyses detected the presence of *trossulus* chimerism in the single individual analysed by this method and otherwise presenting an apparent total remission. If we consider only individuals tested negative at the first haemolymph drawing (n=53), the heavy intensity stage was usually reached 6-9 weeks after the first
neoplastic cells appeared. However, in a few individuals, the course of the disease was more acute: in six individuals the process lasted 3 weeks and in two others, after having been diagnosed negative during the last haemolymph drawing, the haemocytological observations carried out at their death 10 and 12 days later revealed an advanced stage of the disease. Interestingly, in all individuals the same neoplastic cell type was observed during all the course of the disease. Except for individuals undergoing remission processes, DN-affected mussels that reached the high intensity stage usually died between about 2-4 weeks later. Moreover, all the individuals diagnosed as high intensity at their arrival died within the first month.

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

4854. Discussion

We studied the haemolymph of 1791 mussels *Mytilus* sp. using cytological analysis. Haemocytes constitute the cellular component of haemolymph and their morphological classification was a prerequisite to further exploring the DN development. However, controversy over their characterisation remains. As in previous studies (Rasmussen et al., 1985; Carballal et al., 1997), we classified the circulating haemocytes of both *M. edulis* and *M. galloprovincialis* specimens in two morphologically distinct cell types: granulocytes and agranulocytes, depending on the presence of cytoplasmic granules. The distinction between two types of granulocytes suggested by Pipe (1990), some with small or large granules, was not univocal in our samples because granule dimension varied in a single cell. Carballal et al. (1997) observed bi/multi-nucleated granulocytes in a mussel parasitized by trematodes. The presence of basophilic granulocytes appeared to be very limited; they were observed in less than 2% of individuals and represented only few cells, but were reported from nearly all sampling sites. Le Foll et al. (2010) reported a higher proportion of basophilic granulocytes in haemolymph (20-25%). We did not observe the granulocytes containing both types of granules (eosinophilic and basophilic) described by Carballal et al. (1997). These differences could be
attributed to different physiological or environmental conditions among the studies. We observed the small and large agranulocytes that Moore and Lowe (1977) suggested considering as two subpopulations. Brown cells were observed in the haemolymph of some individuals, corresponding morphologically to the brown cells described in mussel tissues by Moore and Lowe (1977) and Carballal et al. (1997). We observed that their presence in the haemolymph was linked to the invasion of bacteria and the number of cells was particularly high in the batch from Lannion, sampled on 20th July during a mortality event. Granular haemocytes were the major constituent of the circulating cell populations. The haemocytograms from mussels of all origins showed a similar pattern with no significant differences except for the two batches sampled in February, both with a lower total haemocyte count. A seasonal evolution in total haemocyte count was previously observed in bivalves, linked to water temperature variations or gonadal development (Soudant et al., 2004; da Silva et al., 2008; Flye-Sainte-Marie et al., 2009; Perrigault et al., 2011) but also to the presence of pathogens (Paris et al., 2008; Ciacci et al., 2009) or pollution (Auffret et al., 2006). Granulocytosis and granulocytopenia were also observed in approximately 4% and 8% of individuals, respectively, but further studies are needed to understand their causes and the mechanisms leading to these conditions. In fact, haemocyte populations might vary greatly with environmental and physiological conditions or in presence of pollutants and pathogens (Pipe and Coles, 1995; Soudant et al., 2004; da Silva et al., 2008; Donaghy and Volety, 2011). By quantifying the mitotic activity of haemolymph cells, Mayrand et al. (2005) showed that blue mussels exposed to a stressor were able to control the number of circulating haemocytes by adjusting the mitotic activity of haemocytes. Contrary to reports by Moore and Lowe (1977) in M. edulis and Matozzo et al. (2008) in Ruditapes decussatus, we did not observe mitotic figures in the small agranular cells but mitoses were observed in granulocytes and, more rarely, in the large agranular cells, questioning the function as stem cells attributed to small agranulocytes. The haematopoiesis in bivalves is still a debated topic, principally due to the lack of specific markers. There are several theories on bivalve haemocyte renewal and maturation (see review Pila et al., 2016). A definitive haematopoietic site has yet to be identified.
Our first goal was to identify the best method to diagnose and follow the evolution of DN. We showed that drawing haemolymph samples was non-destructive and individuals can be repeatedly sampled without affecting survival, as also demonstrated in *Mya arenaria* (Brown et al., 1978; Cooper et al., 1982). This finding is relevant for future studies on mussel haemolymph. We showed that the sensitivity of haemocytological observation appears to be appropriate for DN diagnosis, comparable to that of histology in early stages of the disease. The relative accuracy of these two approaches is most likely a function of the relative amount of tissue that is processed and examined. Haemocytological observation detected one neoplastic cell among about 150,000 fixed on the slide. Flow cytometry and genetics appeared to be reasonable diagnostic methods but might not be sufficiently sensitive in early stages of the disease. However, genetic analysis is the only method that can diagnose neoplasia as a *trossulus* transmissible cancer. Although the fluorescence signal is likely too low for lightly infected specimens, it remains possible that some neoplasms were not the *trossulus* transmissible cancer.

Neoplastic cells were easily identifiable by haemocytology and histology because of their peculiar morphology compared to normal haemocytes. They were characterised by a larger diameter, higher nuclear-cytoplasm ratio, prominent nucleoli and marked basophilia. Histological observation revealed that, in tissues, the neoplastic cell population within a specimen was heteromorphic, confirming the observations of Carella et al. (2013). In contrast, the population circulating in the haemolymph appeared to be relatively homogeneous. Among mussels with DN, four types of anomalous cells were observed in the haemolymph (P-type, Ps-type, U-type, and S-type) but usually only one type was detected in a single individual. These cell-types clearly differed in size, NCR, nucleus shape, number of nucleoli, and basophilia. The P-type was the most frequent form of neoplasia. Previous studies, conducted on other bivalve species, described only two neoplastic circulating cell-types, type A and type B (Farley and Sparks, 1970; Lowe and Moore, 1978; Green and Alderman, 1983) that correspond only partially to P-type and U-type of our study. We did not find a link between cell type and disease stage, thus it is unlikely that the various cell types correspond to a continuum of differentiation. Cells undergoing mitosis and plurinucleated cells were common in tissues, as reported in previous studies (Mix et al., 1979; Carella et al., 2013) but were rare among the circulating cells.
Neoplastic cells were also easily distinguishable by flow cytometry due to their high DNA content, ranging between about 8 and 18 times the normal haploid DNA content, depending on the individual. Clearly, a relation between the nucleus size (measured by haemocytological observation) and ploidy was established allowing a predictive estimation of the DNA content. The low coefficient of variation in fluorescence values among the neoplastic cells of a single mussel showed that the pattern of DNA content of this population within the host was uniform with a comparable ploidy in all the circulating anomalous cells. Only one individual presented simultaneously two neoplastic cell populations, distinct for their ploidy but not enough to be distinguishable morphologically. The high DNA content of neoplastic cells in mussels was mentioned in previous reports, even when different degrees of ploidy were reported. Interestingly, we observed the absence of correlation between the disease stage and ploidy value. This conclusion contrasted with the results of Benabdelmouna et al. (2018) in mussels and Le Grand et al. (2010) in cockles, who suggested an evolutive continuum of ploidies during disease development. However, these two studies did not use genetic analysis, which might have determined if they were describing the transmissible neoplasia we studied. Our results are more consistent with the observations of neoplasia in *M. trossulus* by Moore et al. (1991) and Elston et al. (1990), which were likely transmissible neoplasias (Metzger, 2016). These authors showed that neoplasia exists in two forms in this host, pentaploid and tetraploid, representing distinct pathogenetic processes rather than sequential stages of a single pathogenesis. In our study concerning *M. edulis* hosts, the definition of disease forms according to ploidy was not so conclusive as a high inter-individual variability existed. Unfortunately, all previous flow cytometry analyses carried out on mussel DN, except the study of Benabdelmouna et al. (2018), concerned *M. trossulus* hosts and therefore the descriptions of the ploidy patterns are limited for *M. edulis* and *M. galloprovincialis*. In our samples, the number of circulating neoplastic cells in the S/G2M phases was very low, to such an extent that no corresponding peak was graphically distinguishable, whereas Elston et al. (1990) and Moore et al (1991) in *M. trossulus*, described a cell population in the S/G2M phases. Our observations in flow cytometry confirmed the low mitotic rate detected by haemocytological observation.
The genetic analysis shows that DN of the French mussels we studied is a *trossulus* transmissible cancer, as proposed by Riquet et al. (2017). *Trossulus* alleles in haemolymph DNA of *M. edulis* hosts were amplified regardless of the type of neoplasia. Although the four DN types might correspond to different lineages of transmissible cancer, our results with 12 diagnostic genetic markers show they all share a *M. trossulus* origin. Further investigations will be required to determine if the different types are sublineages of the same emergence or independent emergences. Of interesting note, no *trossulus* individuals have been identified previously in France. Questions remain regarding how and when this neoplasm colonized *M. edulis* mussels in France from its *M. trossulus* population of origin: (i) naturally by stepping stone propagation from *M. trossulus* populations, and it has been missed until now or (ii) by recent anthropogenic induced migration.

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

We detected disseminated neoplasia in locations affected by higher mortality as well as in mortality-free sites (Bernard et al., 2017, 2018; REPAMO https://wwz.ifremer.fr/sante_mollusques), and DN was not detected in the Daoulas site in May 2018 during an event of high mortality. This site regularly has higher than normal mortality but DN was rare (only one positive of the 300 mussels tested). However, 23% of the individuals from this batch showed a supplementary population of nearly triploid cells during flow cytometry analysis while no anomalies were observed in haemocytological observation. A similar condition with a second slightly hyperdiploid peak was observed in *M. trossulus* by Elston et al. (1990), who suggested it to be a distinct normal cell population. In the same Daoulas batch, other 20% of individuals presented with more than 10% of circulating haemocytes in
the S-G2/M phases. This kind of genomic abnormality, the origin of which is unknown but may be promoted by contaminants, was linked to mortalities by Benabdelmouna and Ledu (2016). Nevertheless, the prevalence of DN was above the mean value of 3.29% in four of the six batches regularly affected by high mortality, but was also far lower than the mortality rate recorded. According to these observations, it was not possible to assign the cause of mortality to DN alone.

Our efforts to anticipate the diagnosis of the disease by identifying early indicators were not conclusive. No correlation between neoplasia and other variables (haemocytogram values, inflammatory lesions such as granulocytomas and haemocytic infiltration, and early genomic abnormalities) was demonstrated. Presently, the disease seems to be diagnosable only when characteristic neoplastic cells appear. However, with the evidence of the genetic peculiarity of neoplastic cells, a molecular test that is rapid, sensitive and specific for DN is likely to be developed in the near future. According to the results of our monitoring, carried out by haemocytological observation of mussels maintained under controlled and stable conditions, disease incidence was not constant over time but followed the same trend in all the batches, reaching a maximum after 2 months and then decreasing. Various interpretations are possible: i) the infection occurred in the natural environment before the collection and was not transmitted under the laboratory conditions, even if at least one DN-affected mussel was always present in each tank; ii) the sensitivity to DN varies among individuals and some of them, the survivors, are resistant; iii) physiological conditions are determinants for the development of the disease. Assuming the first hypothesis is supported, it would mean that we were not able to detect the first stage of infection for months and suggests the existence of a quiescent state. Disease progression in affected mussels appeared usually rather slow to reach the complete replacement of normal haemocytes by neoplastic cells, followed by death about 1 month later. Disease progression is, however, poorly generalisable. In fact, in few cases, we observed a rapid and total invasion of haemolymph by neoplastic cells in less than 2 weeks, probably promoted by stress factors or due to a more aggressive DN form. Unfortunately, because our monitored batches suffered mortality not attributable to DN, we were not able to evaluate consistently whether differences exist between the various forms of neoplasia. Finally, remission of DN was observed and
this proved to be quite frequent (12%), regardless of the neoplasia type. A higher remission rate (20%) was observed in *M. trossulus* (Elston et al., 1988), confirming that it is common in the course of DN infection. The rapid decrease of the number of circulating anomalous cells during remission was also observed in tissues. Interestingly, the DNA of neoplastic cells was still detectable after complete remission by genetic analysis. Two assumptions can be made i) *trossulus* DNA resulting from the destruction of neoplastic cells is still present, free or within phagosomes, ii) *trossulus* cells are still present in the haemolymph but are undistinguishable from host haemocytes at this stage.

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

**5. Conclusions**

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

Some observations raised new questions:
We described the normal cellular composition of the haemolymph of blue mussels and detected that some variations occur. Further studies are needed to understand the origin, the consequences, and the function of these alterations.

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

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

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

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

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