



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

Erika A.V. Burioli, Suzanne Trancart, Alexis Simon, I. Bernard, Maud Charles, Elise Oden, Nicolas Bierne, Maryline Houssin

► To cite this version:

Erika A.V. Burioli, Suzanne Trancart, Alexis Simon, I. Bernard, Maud Charles, et al.. Implementation of various approaches to study the prevalence, incidence and progression of disseminated neoplasia in mussel stocks. *Journal of Invertebrate Pathology*, 2019, 168, pp.107271. 10.1016/j.jip.2019.107271 . hal-02404031

HAL Id: hal-02404031

<https://hal.science/hal-02404031>

Submitted on 25 Nov 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

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

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

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

5¹ LABÉO, 1 Route de Rosel, 14280 St Contest, France

6² Institut des Sciences de l'Évolution UMR5554, CNRS-IRD-EPHE-UM, Route de Mende, 34090 Montpellier,
7France

8³ Eurêka Modélisation, 13 rue de Kermarquer, Lézardrieux, France

9⁴ Université de Caen Normandie, UMR BOREA, MNHN, UPMC, UCBN, CNRS-7208, IRD-207, Esplanade de
10la paix, 14000 Caen, France

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)

82associated abnormal outbreaks of mussel mass mortality with poor cytogenetic quality, particularly in
83terms of ploidy variations in circulating cells. In a second study, Benabdelmouna et al. (2018) linked
84these genomic abnormalities to ongoing neoplastic processes. DN was also suspected to be involved in
85these 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
87economically important bivalve stocks have occasionally been linked to high prevalence of DN in
88other parts of the world (Frierman and Andrews, 1976; Elston et al., 1992; McGladdery et al., 2001;
89Villalba, et al., 2001).

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

97

982. Materials and Methods

992.1. Sampling

100Eighteen batches of mussels were collected during sampling campaigns conducted between July 2017
101and 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
103wild in the prior year before being moved to their final growing sites. At time of sampling, individuals
104from 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-
106Coutainville, St Brieuc, Daoulas, and Pénestin) or in suspension in deep waters (Lannion and
107Camaret). 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/), 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 μ 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.

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 cytospin slide 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 basophily. 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

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

When flow cytometry analysis was conducted on mussel individuals at the same time as cytological analysis, a supplementary volume of 100 μ L haemolymph was drawn and placed in a 1.5-mL microtube; 500 μ L 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 μ L 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 μ L 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 μ L/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- μ 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.

242 2.8. 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

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 $4.4 (\pm 1.3) \times 10^6$ cells/mL but, in the individuals sampled in February, haemocyte count was significantly lower, mean $9.8 (\pm 1.2) \times 10^5$ cells/mL (ANOVA, $p < 0.05$), in the three sampled sites, without significant inter-individual variations. The proportion of granulocytes was mean $64 (\pm 4.3) \%$. 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 $>8 \times 10^6$ 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 (2.8×10^6 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.

3.3 *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.

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_2M (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
379 $33.12(\pm 10.02)\%$ 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-G₂/M phase.
394 We never observed a second peak on the cytometry histogram of the DN-affected individuals that
395 could represent the G₂/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-G₂/M phase
399 compared with the other sites (Kruskal-Wallis, $K=100.57$, $df=5$, $p < 2.2e-16$), representing
400 $7.45(\pm 3.56)\%$ vs $2.51(\pm 1.92)\%$, respectively. Moreover, in 19.54% of the individuals from Daoulas,
401 more than 10% of circulating haemocytes were in the S-G₂/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

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 23.29%. 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 ($\chi^2_{(1,0.05)}=53.16$, $p\text{-value}=3.07\times10^{-13}$). 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

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 26th July and 1st August 2017, respectively, had comparable prevalence. Significant
449differences (χ^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 (χ^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

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.

4. 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

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 20th
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; Ciacchi 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.

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.

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), 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

675 Acknowledgements

676 We express our gratitude to the farmers for supplying mussel samples. This work received financial
677 support from the “Conseil Régional de Normandie”. We thank the Comité Régional de la
678 Conchyliculture de Bretagne Nord (CRC). A. S. and N. B. acknowledge funding by Montpellier
679 Université d’Excellence (BLUECANCER project) and Agence Nationale de la Recherche
680 (TRANSCAN project, ANR-18-CE35-0009).

681

682 References

683 AboElkhair, M., Iwamoto, T., Clark, K.F., McKenna, P., Siah, A., Greenwood, S.J., Berthe, F.C.J.,
684 Casey, J.W., Cepica, A., 2012. Lack of detection of a putative retrovirus associated with haemic

685 neoplasia in the soft-shell clam *Mya arenaria*. J. Invertebr. Pathol., 109: 97-104. doi:
686 10.1016/j.jip.2011.10.008.

687 AboElkhair, M., Siah, A., Clark, K.F., McKenna, P., Pariseau, J., Greenwood, S.J., Berthe, F.C.J.,
688 Cepica, A., 2009. Reverse transcriptase activity associated with haemic neoplasia in the soft-
689 shell clam *Mya arenaria*. Dis. Aquat. Org., 84: 57-63. doi: 10.3354/dao02038.

690 Auffret, M., Poder, M., 1986. Sarcomatous lesion in the cockle *Cerastoderma edule*. II. Electron
691 microscopy study. Aquaculture, 58: 9-15. doi: 10.1016/0044-8486(86)90152-3.

692 Auffret, M., Rousseau, S., Boutet, I., Tanguy, A., Baron, J., Moraga, D., Duchemin, M.B., 2006. A
693 multiparametric approach for monitoring immunotoxic responses in mussels from contaminated
694 sites in Western Mediterranean. Ecotoxicology and Environmental Safety 63(3): 393-405. doi:
695 10.1016/j.ecoenv.2005.10.016.

696 Balouet, G., Poder, M., Cahour, A., Auffret, M., 1978. Proliferative hemocytic condition in European
697 flat oysters (*Ostrea edulis*) from Breton coasts: A 6-year survey. J. Invert. Pathol., 48(2): 208-
698 215. doi: 10.1016/0022-2011(86)90125-4.

699 Barber, B.J., 2004. Neoplastic diseases of commercially important marine bivalves. Aquat. Living
700 Resour. 17, 449-466. doi: 10.1051/alr:2004052.

701 Bayne, B.L., 1976. Marine mussels, Their Ecology and Physiology. The University Press, Cambridge,
702 MA, 506 pp.

703 Béchemin, C., Soletchnik, P., Polsenaere, P., Le Moine, O., Pernet, F., Protat, M., Fuhrman, M., Quéré,
704 C., Goulitquer, S., Corporeau, C., Lapègue, S., Travers, A., Morga, B., Garrigues, M., Garcia C.,
705 Haffner, P., Dubreuil, C., Faury, N., Baillon, L., Baud, J.-P., Renault, T., 2015. Episodes de
706 mortalité massive de moules bleues observés en 2014 dans le Pertuis charentais. Bull.
707 épidémiol., santé anim. aliment., 67: 6-9.

708 Ben Cheikh, Y., Travers, M. A., Morga, B., Godfrin, Y., Rioult, D., Le Foll, F., 2016. First evidence for
 709 a *Vibrio* strain pathogenic to *Mytilus edulis* altering hemocyte immune capacities.
 710 Developmental and Comparative Immunology, 57: 107-119. doi: 10.1016/j.dci.2015.12.014.

711 Ben Cheikh, Y., Travers, M. A., Le Foll, F., 2017. Infection dynamics of a *V. splendidus* strain
 712 pathogenic to *Mytilus edulis*: In vivo and in vitro interactions with hemocytes. Developmental
 713 and Comparative Immunology, 70: 515-523. doi: 10.1016/j.fsi.2017.09.047.

714 Benabdelmouna, A., Ledu, C., 2016. The mass mortality of blue mussels (*Mytilus* spp.) from the
 715 Atlantic coast of France is associated with heavy genomic abnormalities as evidenced by flow
 716 cytometry. J. Invertebr. Pathol., 138: 30-38. doi: 10.1016/j.jip.2016.06.001.

717 Benabdelmouna, A., Saunier, A., Ledu, C., Travers, M.A., Morga, B., 2018. Genomic abnormalities
 718 affecting mussels (*Mytilus edulis-galloprovincialis*) in France are related to ongoing neoplastic
 719 processes, evidenced by dual flow cytometry and cell monolayer analyses. J. Invertebr. Pathol.,
 720 157: 45-52. doi: 10.1016/j.jip.2018.08.003.

721 Bernard, I., Allain, G., 2017. Mortalités des moules en Bretagne nord : bilan des connaissances. Rapp.
 722 tech. CRC Bretagne nord. 37 pp.

723 Bernard, I., Allain, G., Charles, M., Villalba, A., Burioli, E., Houssin, M., Deydier, Y., 2017.
 724 Investigation on blue mussel high mortality in France: a phenomenon with high spatio-temporal
 725 variability but no conclusive cause. AMMR 2017, 2nd International Symposium on the
 726 Advances in Marine Mussel Research, 7th & 8th of September 2017, Sète (France).

727 Bernard, I., Charles, M., Allain, G., Burioli, E., Villalba, A., Foll, F. L., Deydier, Y., Houssin, M.,
 728 2018. Bilan de l'observatoire des mortalités de moules en Bretagne Nord pour la saison 2016-
 729 2017 et premiers éléments sur les organismes pathogènes présents. Rapp. tech. CRC Bretagne
 730 Nord. 26 pp.

731Bierne, N., Borsa, P., Daguin, C., Jollivet, D., Viard, F., Bonhomme, F., David, P., 2003. Introgression
 732 patterns in the mosaic hybrid zone between *Mytilus edulis* and *M. galloprovincialis*. Mol. Ecol.,
 733 12(2): 447-461. doi: 10.1046/j.1365-294X.2003.01730.x.

734Brown, R.S., Wolke, R.E., Saila, S.B., Brown, C.W., 1978. Prevalence of neoplasia in 10 New
 735 England populations of the soft-shell clam (*Mya arenaria*). Proc. Conf. on Aquatic Pollutants
 736 and Biologic Effects with Emphasis on Neoplasia. Ann. N.Y. Acad. Sci., 298: 522-534. doi:
 737 10.1111/j.1749-6632.1977.tb19287.x.

738Burioli, E.A.V., Charles, M., Bernard, I., Pitel, P.H., Houssin, M., 2017. First description of
 739 disseminated neoplasia in *Mytilus edulis* in Northern Brittany (France) and development of a
 740 rapid diagnostic tool. AMMR 2017, 2nd International Symposium on the Advances in Marine
 741 Mussel Research, 7th & 8th of September 2017, Sète (France). doi:
 742 10.13140/RG.2.2.10832.20488.

743Carballal, M.J., Barber B.J., Iglesias, D., Villalba, A., 2015. Neoplastic diseases of marine bivalves. J.
 744 Invertebr. Pathol., 131: 83-106. doi: 10.1016/j.jip.2015.06.004.

745Carballal, M.J., López, M.C., Azevedo, C., Villalba, A., 1997. Hemolymph cell types of the mussel
 746 *Mytilus galloprovincialis*. Dis. Aquat. Org., 29: 127-135.

747Carella, F., De Vico G., Landini, G., 2017. Nuclear morphometry and ploidy of normal and neoplastic
 748 haemocytes in mussels. PLoS ONE 12(3): e0173219. doi: 10.1371/journal.pone.0173219.

749Carella, F., Figueras, A., Novoa, B., De Vico, G., 2013. Cytomorphology and PCNA expression
 750 pattern in bivalves *Mytilus galloprovincialis* and *Cerastoderma edule* with haemic neoplasia.
 751 Dis. Aquat. Org., 105: 81-87. doi: 10.3354/dao02612.

752Ciacci, C., Citterio, B., Betti, M., Canonico, B., Roch, P., Canesi, L., 2009. Functional differential
 753 immune responses of *Mytilus galloprovincialis* to bacterial challenge. Comp. Biochem. Physiol.
 754 Biochem. Mol. Biol, 153(4): 365-71. doi: 10.1016/j.cbpb.2009.04.007.

755 Collins, C.M., Mulcahy, M.F., 2003. Cell-free transmission of a haemic neoplasm in the cockle
756 *Cerastoderma edule*. Dis. Aquat. Organ., 54(1): 61-67. doi: 10.3354/dao054061.

757 Cooper, K.R., Brown, R.S., Chang, P.W., 1982. Accuracy of blood cytological screening techniques
758 for the diagnosis of a possible hematopoietic neoplasm in the bivalve mollusc, *Mya arenaria*. J.
759 Invertebr. Pathol., 39: 281-289.

760 Cuenca, J., Aleza, P., Navarro, L., Ollitrault, P., 2013. Assignment of SNP allelic
761 configuration in polyploids using competitive allele-specific PCR: application
762 to citrus triploid progeny. Ann. Bot., 111(4), 731–742. doi:
763 10.1093/aob/mct032.

764 da Silva, P.C., Comesaña, P., Fuentes, J., Villalba, A., 2008. Variability of haemocyte and haemolymph
765 parameters in European flat oyster *Ostrea edulis* families obtained from brood stocks of
766 different geographical origins and relation with infection by the protozoan *Bonamia ostreae*.
767 Fish Shellfish Immunol., 24: 551-563. doi: 10.1016/j.fsi.2007.11.003.

768 da Silva, P.M., Soudant, P., Carballal, M.J., Lambert, C., Villalba, A., 2005. Flow cytometric DNA
769 content analysis of neoplastic cells in haemolymph of the cockle *Cerastoderma edule*. Dis.
770 Aquat. Organ. 67, 133-139. doi: 0.3354/dao067133.

771 Delaporte, M., Synard, S., Pariseau, J., McKenna, P., Tremblay, R., Davidson, J., Berthe, F.C., 2008.
772 Assessment of haemic neoplasia in different soft-shell clam *Mya arenaria* populations from
773 eastern Canada by flow cytometry. J. Invertebr. Pathol. 98, 190-197. doi :
774 10.1016/j.jip.2007.12.005.

775 Díaz, S., Villalba, A., Insua, A., Soudant, P., Fernandez-Tajes, J., Mendez, J., Carballal, M.J., 2013.
776 Disseminated neoplasia causes changes in ploidy and apoptosis frequency in cockles
777 *Cerastoderma edule*. J. Invertebr. Pathol. 113, 214-219. doi: 10.1016/j.jip.2013.03.010.

778 Donaghy, L., Volety, A.K., 2011. Functional and metabolic characterization of hemocytes of the green
779 mussel, *Perna viridis*: *in vitro* impacts of temperature. Fish Shellfish Immunol., 31: 808-814.
780 doi: 10.1016/j.fsi.2011.07.018.

781 Elston, R.A., Drum, A.S., Allen, S.K.Jr., 1990. Progressive development of circulating polyploid cells
 782 in *Mytilus* with hemic neoplasia. Dis. Aquat. Org., 8: 51-59.

783 Elston, R.A., Kent M.L., Drum, A.S., 1988. Transmission of hemic neoplasia in the bay mussel,
 784 *Mytilus edulis*, using whole cells and cells homogenate. Dev. Comp. Immunol., 12: 719-727.
 785 doi: 10.1016/0145-305X(88)90047-X.

786 Elston, R.A., Moore, J.D., Brooks, K., 1992. Disseminated neoplasia of bivalve molluscs. Rev. Aquat.
 787 Sci. 6, 405-466.

788 FAO, Food and Agriculture Organisation of the United Nations (2016). Fisheries and Aquaculture
 789 Department, Dataset Global Aquaculture production. On-Line Query.

790 Farley, C.A., 1969a. Probable neoplastic disease of the hematopoietic system in oysters, *Crassostrea*
 791 *virginica* and *Crassostrea gigas*. Natl. Cancer I. Monogr. 31: 541-555.

792 Farley, C.A., 1969b. Sarcomatoid proliferative disease in a wild population of blue mussels (*Mytilus*
 793 *edulis*). Natl. Cancer I. Monogr., 43: 509-516.

794 Farley, C.A., Sparks, A.K., 1970. Proliferative diseases of hemocytes, endothelial cells, and connective
 795 tissue cells in mollusks. Bibl. Haematol. 36: 610-617.

796 Flye-Sainte-Marie, J., Soudant, P., Lambert, C., Le Goïc, N., Goncalvez, M., Travers, M.A., Paillard,
 797 C., Jean, F., 2009. Variability of the hemocyte parameters of *Ruditapes philippinarum* in the
 798 field during an annual cycle. J. Exp. Marine Biol. Ecol., 377: 1-11. doi:
 799 10.1013/j.embe.2009.06.003.

800 Fraïsse, C., Belkhir, K., Welch, J.J., Bierne, N., 2016. Local interspecies introgression is the main
 801 cause of extreme levels of intraspecific differentiation in mussels. Mol. Ecol., 25(1), 269–286.
 802 doi: 10.1111/mec.13299.

803 Frierman, E.M., Andrews, J.D., 1976. Occurrence of haematopoietic system in oysters, *Crassostrea*
 804 *virginica* and *Crassostrea gigas*. J. Natl. Cancer I., 56: 319-324. 10.1093/jnci/56.2.319.

805 Galimany, E., Sunila, I., 2008. Several cases of disseminated neoplasia in mussels *Mytilus edulis* (L.)
806 in western long island sound. J. Shellfish Res. 27(5), 1201-1207. doi: 10.2983/0730-8000-
807 27.5.1201.

808 Gombač, M., Sitar, R., Pogačnik, M., Arzul, I., Jenčič, V., 2013. Haemocytic neoplasia in
809 Mediterranean mussels (*Mytilus galloprovincialis*) in the Slovene Adriatic Sea. Mar. Freshwater
810 Behav. Physiol., 46(2): 135-143. doi: 10.1080/10236244.2013.782736.

811 González-Tizón, A.M., Martínez-Lage, A., Rego, I., Ausió, J., Méndez J., 2000. DNA content,
812 karyotypes, and chromosomal location of 18S-5.8S-28S ribosomal loci in some species of
813 bivalve molluscs from the Pacific Canadian coast. Genome, 43(6): 1065-1072. doi:
814 10.1139/g00-089.

815 Green, M., Alderman, D.J., 1983. Neoplasia in *Mytilus edulis* L. from United Kingdom waters.
816 Aquaculture, 30(1-4):1-10. doi: 10.1016/0044-8486(83)90146-1.

817 House, M.L., 1997. Transmission of disseminated neoplasia in the soft-shell clam, *Mya arenaria*. PhD
818 thesis, Oregon State University, Corvallis.

819 House, M.L., Kim, C.H., Reno, P.W., 1998. Soft shell clams *Mya arenaria* with disseminated
820 neoplasia demonstrate reverse transcriptase activity. Dis. Aquat. Org., 34(3): 187-192. doi:
821 10.3354/dao034187.

822 Inoue, K., Waite, J.H., Matsuoka, M., Odo, S., Harayama, S., 1995. Interspecific variations in adhesive
823 protein sequences of *Mytilus edulis*, *M. galloprovincialis*, and *M. trossulus*. Biol. Bull.
824 189(3):370-375. doi: 10.2307/1542155.

825 Kent, M.L., Wilkinson, M.T., Drum, A.S., Elston, R.A., 1991. Failure of transmission of hemic
826 neoplasia of bay mussels, *Mytilus trossulus*, to other bivalves. J. Invertebr. Pathol., 57: 435-436.

827 Krishan, A., 1975. Rapid cytofluorometric analysis of mammalian cell cycle by propidium iodide
828 staining. J. Cell. Biol., 66:188–193. doi: 10.1083/jcb.66.1.188.

829 Landsberg, J.H., 1996. Neoplasia and biotoxins in bivalves: is there a connexion? J. Shellfish Res. 15,
830 203-230.

831 Le Foll, F., Rioult, D., Boussa, S., Pasquier, J., Dagher, Z., Leboulenger, F., 2010. Characterisation of
832 *Mytilus edulis* hemocyte subpopulations by single cell time-lapse motility imaging. Fish
833 Shellfish Immunol., 28(2):372-86. doi: 10.1016/j.fsi.2009.11.011.

834 Le Grand, F., Kraffe, E., de Montaudouin, X., Villalba, A., Marty, Y., Soudant, P., 2010. Prevalence,
835 intensity, and aneuploidy patterns of disseminated neoplasia in cockles (*Cerastoderma edule*)
836 from Arcachon Bay: seasonal variation and position in sediment. J. Invertebr. Pathol. 104, 110-
837 118. doi: 10.1016/j.jip.2010.01.011.

838 Lowe, D.M., Moore, M.N., 1978. Cytology and quantitative cytochemistry of a proliferative atypical
839 hemocytic condition in *Mytilus edulis* (Bivalvia, Mollusca). J. Natl. Cancer I., 60(6):1455-1459.
840 doi: 10.1093/jnci/60.6.1455.

841 Lowe, D.M., Moore, M.N., 1979. The cytology and occurrence of granulocytomas in mussels. Mar.
842 Pollut. Bull., 10: 137-141. doi: 10.1016/0025-326X(79)90081-X.

843 Matozzo, V., Marin, M.G., Cima, F., Ballarin, L., 2008. First evidence of cell division in circulating
844 haemocytes from the Manila clam *Tapes philippinarum*. Cell Biol. Intern., 32: 865-868. doi:
845 10.1016/j.cellbi.2008.03.008.

846 Mayrand, E., St-Jean, S.D., Courtenay, S.C., 2005. Haemocyte responses of blue mussels (*Mytilus*
847 *edulis* L.) transferred from a contaminated site to a reference site: can the immune system
848 recuperate? Aquaculture Res., 36: 962-971. doi: 10.1111/j.1365-2109.2005.01302.x.

849 McGladdery, S.E., Reinisch, C.L., MacCallum, G.S., Stephens, R.E., Walker, C.L., Davidson, J.T.,
850 2001. Haemic neoplasia in soft-shell clams *Mya arenaria*: recent outbreaks in Atlantic Canada
851 and discovery of a p53 gene homologue associated with the condition. Bull. Aquat. Assoc. Can.,
852 101, 19-26.

853McLaughlin, S.M., Farley, C.A., Hetrick, F.M., 1992. Transmission studies of sarcoma in the soft-shell
854 clam, *Mya arenaria*. In Vivo, 6: 367-370.

855Metzger, M.J., Reinisch, C., Sherry, J., Goff, S.P., 2015. Horizontal transmission of clonal cancer cells
856 causes leukemia in soft-shell clams. Cell, 161: 255-263. doi: 10.1016/j.cell.2015.02.042.

857Metzger, M.J., Villalba, A., Carballal, M.J., Iglesias, D., Sherry, J., Reinisch, C., Muttray, A.F.,
858 Baldwin, S.A., Goff, S.P., 2016. Widespread transmission of independent cancer lineages within
859 multiple bivalve species. Nature, 534: 705–709. doi: 10.1038/nature18599.

860Mix, M.C., Hawkes, J.W., Sparks, A.K., 1979. Observations of the ultrastructure of large cells
861 associated with putative neoplastic disorders of mussels, *Mytilus edulis*, from Yaquina Bay,
862 Oregon. J. Invertebr. Pathol., 34: 41-56. doi: 10.1016/0022-2011(79)90052-1.

863Moore, J.D., Elston, R.A., Drum, A.S., Wilkinson, M.T., 1991. Alternate pathogenesis of systemic
864 neoplasia in the bivalve mollusc *Mytilus*. J. Invertebr. Pathol., 58: 231-243. doi: 10.1016/0022-
865 2011(91)90067-Z.

866Moore, M.N., Lowe, D.M., 1977. The cytology and cytochemistry of the hemocytes of *Mytilus edulis*
867 and their response to experimentally injected carbon particles. J. Invertebr. Pathol., 29: 18-30.
868 doi: 10.1016/0022-2011(77)90167-7.

869Oden, E., Burioli, E.A.V., Trancart, S., Pitel, P.H., Houssin, M., 2016. Multilocus Sequence Analysis of
870 *Vibrio splendidus* related-strains isolated from blue mussel *Mytilus* sp. during mortality events.
871 Aquaculture, 464: 420-427. doi: 10.1016/j.aquaculture.2016.07.024.

872Oprandy, J.J., Chang, P.W., 1983. 5-bromodeoxyuridine induction of hematopoietic neoplasia and
873 retrovirus activation in the soft-shell clam, *Mya arenaria*. J. Invertebr. Pathol., 42: 196-206. doi:
874 10.1016/0022-2011(83)90062-9.

875Oprandy, J.J., Chang, P.W., Pronovost, A.D., Cooper, K.R., Brown, R.S., Yates, V.J., 1981. Isolation of
876 a viral agent causing haematopoietic neoplasia in the soft-shell clam *Mya arenaria*. J. Inverteb.
877 Pathol., 38(1): 45-51. doi: 10.1016/0022-2011(81)90033-1.

878Parisi, M.G., Li, H., Jouvett, L.B.P., Dyrynda, E.A., Parrinello, N., Cammarata, M., Roch, P., 2008.
879 Differential involvement of mussel hemocyte sub-populations in the clearance of bacteria. Fish
880 Shellfish Immunol., 25: 834-840. doi: 10.1016/j.fsi.2008.09.005.

881Perrigault, M., Dahl, S.F., Pales Espinosa, E., Gambino, L., Allam, B., 2011. Effects of temperature on
882 hard clam (*Mercenaria mercenaria*) immunity and QPX (Quahog Parasite Unknown) disease
883 development: II. Defence parameters. J. Invertebr. Pathol., 106: 322-332. doi:
884 10.1016/j.jip.2010.11.004.

885Pila, E.A., Sullivan, J.T., Wu, X.Z., Fang, J., Rudko, S.P., Gordy, M.A., Hanington, P.C., 2016.
886 Haematopoiesis in molluscs: A review of haemocyte development and function in gastropods,
887 cephalopods and bivalves. Dev. Comp. Immunol., 58: 119-128. doi: 10.1016/j.dci.2015.11.010.

888Pipe, R.K., 1990. Differential binding of lectins to haemocytes of the mussel *Mytilus edulis*. Cell
889 Tissue Res., 261: 261:268. doi: 10.1007/BF00318667.

890Pipe, R.K., Coles, J.A., 1995. Environmental contaminants influencing immune function in marine
891 bivalve molluscs. Fish Shellfish Immunol., 5: 581-595. doi: 10.1016/S1050-4648(95)80043-3.

892Polsenaere, P., Soletchnik, P., Le Moine, O., Gohin, F., Robert, S., Pépin, J. F., Stanisière, J. Y.,
893 Dumas, F., Béchemin, C., Goulletquer, P., 2017. Potential environmental drivers of a regional
894 blue mussel mass mortality event (winter of 2014, Breton Sound, France). J. Sea Res., 123: 39-
895 50. doi: 10.1016/j.seares.2017.03.005.

896Rasmussen, L.P.D., Hage, E., Karlog, O., 1985. An Electron Microscope Study of the Circulating
897 Leucocytes of the Marine Mussel, *Mytilus edulis*. J. Invertebr. Pathol., 45: 158-167. doi:
898 10.1016/0022-2011(85)90005-9.

899Reinisch, C.L., Charles, A.M., Stone, A.M., 1984. Epizootic neoplasia in soft shell clams collected
900 from New Bedford Harbor. Hazardous waste, 1(1): 73-81. doi: 10.1089/hzw.1984.1.73.

901Riquet, F., Simon, A., Bierne, N., 2017. Weird genotypes? Don't discard them, transmissible cancer
902 could be an explanation. Evol. Appl., 10: 140-145. doi: 10.1111/eva.12439.

903 Romalde, J.L., Luz Vilariño, M., Beaz, R., Rodríguez, J.M., Díaz, S., Villalba, A., Carballal, M.J.,
 904 2007. Evidence of retroviral etiology for dissminated neoplasia in cockles (*Cerastoderma*
 905 *edule*), J. Invertebr. Pathol., 94: 95-101. doi: 10.1016/j.jip.2006.09.007.

906 Semagn, K., Babu, R., Hearne, S., Olsen, M., 2014. Single nucleotide polymorphism genotyping using
 907 Kompetitive Allele Specific PCR (KASP): overview of the technology and its application in
 908 crop improvement. Mol. Breeding, 33:1–14. doi: 10.1007/s11032-013-9917-x.

909 Simon, A., Bierne, N., Welch, J. J., 2018. Coadapted genomes and selection on hybrids: Fisher's
 910 geometric model explains a variety of empirical patterns. Evolution Letters, 2(5): 472-498. doi:
 911 10.1002/evl3.66.

912 Soudant, P., Paillard C., Choquet, G., Lambert, C., Reid, H.I., Marhic, A., Donaghy, L., Birkbeck,
 913 T.H., 2004. Impact of season and rearing site on the physiological and immunological
 914 parameters of the Manila clam *Venerupis* (=Tapes, =*Ruditapes*) *philippinarum*. Aquaculture;
 915 229: 401-418. doi: 10.1016/S0044-8486(03)00352-1.

916 Suquet, M., de Kermoysan, G., Gonzalez Araya, R., Queau, I., Lebrun, L., Le Souchu, P., Mingant, C.
 917 (2009). Anesthesia in Pacific oyster *Crassostrea gigas*. Aquat. Living Resour., 22, 29–34. doi:
 918 10.1051/alr/2009006.

919 Twomey, E., Mulcahy, M.F., 1988. Transmission of a sarcoma in the cockle *Cerastoderma edule*
 920 (Bivalvia: Mollusca) using cell transplants. Dev. Comp. Immunol., 12:195-200.

921 Vassilenko, E., Baldwin, S.A., 2014. Using flow cytometry to detect haemic neoplasia in mussels
 922 (*Mytilus trossulus*) from the Pacific Coast of Southern British Columbia, Canada. J. Invertebr.
 923 Pathol., 117: 68-72. doi: 10.1016/j.jip.2014.02.002.

924 Villalba, A., Carballal, M.J., López, C., 2001. Disseminated neoplasia and large foci indicating heavy
 925 haemocytic infiltration in cockles *Cerastoderma edule* from Galicia (NW Spain). Dis. Aquat.
 926 Org. 46: 213-216. doi: 10.3354/dao046213.

927 Weinberg, J.R., Leavitt, D.F., Lancaster, B.A., Capuzzo, J.M., 1997. Experimental field studies with
928 *Mya arenaria* (Bivalvia) on the induction and effect of hematopoietic neoplasia. J. Invertebr.
929 Pathol., 61: 253-259. doi: 10.1006/jipa.1996.4641.
930
931