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Histopathology of mussels (sp.) from the Tamar estuary, UK

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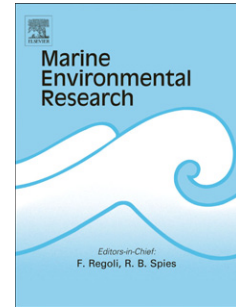
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1 **Histopathology of mussels (*Mytilus* sp.) from the Tamar estuary, UK.**

2

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24 Running title: Histopathology of mussels

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1 **Abstract**

2 This study assessed numerous histological parameters as markers of health status in
3 mussels (*Mytilus* spp.) collected from several locations along a contamination gradient on the
4 River Tamar and two coastal sites on the north and south coast of Cornwall, UK. Twenty-
5 seven health parameters were assessed including the presence of pathogens, inflammatory
6 lesions, non-specific pathologies and reproductive condition. Logistic regression showed that
7 reproductive condition and inflammatory lesions showed changes indicative of a
8 contamination gradient response. Mussel populations further upstream within the Tamar were
9 relatively less developed compared to mussels situated downstream in the estuary.
10 Compared to coastal locations, mussels sampled within the Tamar also exhibited a higher
11 prevalence of inflammatory lesions that increased at locations situated furthest upstream.
12 Similarly, levels of lipofuscin within the kidney epithelium were highest in mussels sampled
13 within the estuary and Whitsand Bay compared to Trebarwith Strand. The most prevalent
14 pathogens observed during this survey included *Rickettsia/Chlamydia*-like organisms
15 (R/CLO), gill ciliates including *Ancistrum mytili*, the copepod *Mytilicola intestinalis*, Ciliophora-
16 like organisms and *Marteilia* sp. *Marteilia* sp. was observed in 31% of the mussels sampled
17 from the Cremyll Ferry site. Generally, the range and prevalence of pathogens was highest
18 from the two estuarine locations at Cremyll Ferry and Wilcove. No significant differences were
19 observed between estuarine sampling sites and Whitsand Bay with respect to median
20 epithelial cell height of digestive tubules, however mussels from Trebarwith Strand did exhibit
21 a reduced median epithelial cell height compared to mussels sampled from other sampling
22 sites. Species genotyping was also undertaken during this study in order to consider any
23 histological differences in relation to species. Results are discussed in relation to
24 histopathology health parameters and biological effects monitoring.

25

26 **Keywords:** Histopathology, biological effects, *Mytilus edulis*, *Mytilus galloprovincialis*,
27 hybrids, diseases, mussels, species.

28

29

30

1 1. Introduction

2
3 Inventory-based chemical monitoring programmes are restricted to the identification of a
4 limited range of contaminants and provide little or no information on their biological
5 significance. Accordingly, increasing emphasis is being placed on determining the causal
6 relationships between contaminant exposure and observable biological effects in aquatic
7 organisms (Thain et al., 2008). It is known that exposure to contaminants are significant
8 stressors for some species, especially in estuaries, and while environmental quality standards
9 in UK marine waters are generally being met they do not take into consideration the effects of
10 chronic exposure to complex mixtures of contaminants (Matthiessen et al., 1993). As such,
11 the identification of fish and shellfish diseases and pathologies, with a broad range of
12 aetiologies, is increasingly being used as indicators of environmental stress since they
13 provide a definite and ecologically-relevant biological end-point of chronic contaminant
14 exposure (Matthiessen et al., 1993; Mix, 1988; Stentiford et al., 2009). Additionally, the
15 application of histological biomarkers provides an effective set of tools for detecting and
16 characterising the biological end points of toxicant exposure (Hinton et al., 1992). Histological
17 lesions have been used as sensitive and reliable indicators to determine the health status of
18 aquatic species in a wide range of studies (Auffret 1988; Stentiford et al., 2009).

19
20 Mussels (*Mytilus* spp) have shown considerable potential as sentinel organisms in studies
21 monitoring the health status of aquatic systems. They are generally abundant, sessile and
22 exhibit a range of biological responses when stressed or exposed to environmental
23 contaminants (Goldberg et al., 1978; Moore et al., 1987; Livingstone and Pipe, 1992;
24 Widdows and Donkin, 1992; Brooks et al., 2009). The application of histopathology to
25 mussels can incorporate measures of reproductive and metabolic condition, and allows for
26 the detection of a range of pathogens that may affect morbidity and mortality. Furthermore,
27 mussel histopathology has been previously employed to investigate the casual relationships
28 between environmental health and exposure to contaminants (Lowe and Pipe, 1987; Auffret,
29 1988; Marigómez et al., 2006; Bignell et al., 2008). As such, a plethora of histopathological
30 criteria can be utilised in monitoring programmes using mussels.

1

2 Here we report prevalence data for a range of pathologies and pathogens present in
3 populations of *Mytilus* spp. collected from the Tamar Estuary, UK and from coastal sites in the
4 South West of England (see Shaw et al.; in this issue). This study was part of an integrated
5 workshop investigating the health status of the Tamar Estuary using a suite of chemical,
6 cellular and whole organism endpoints.

7

8 **2. Material and methods**

9

10 *2. 1. Sampling*

11 As part of the sampling protocol, samples were obtained for analysis of biochemistry, heavy
12 metals, histochemistry, toxicogenomics and biomarker response (Money et al.; Shaw et al., in
13 this issue). Further samples were also obtained for histopathology. Mussels were collected
14 from four sampling sites in and around the Tamar Estuary, South West England (Neal Point,
15 Wilcove, Cremyll Ferry, Whitsand Bay). Samples were also collected from another coastal
16 site (Trebarwith Strand) on the north coast of Cornwall, UK (Figure 1).

17

18 For histopathological analysis of health status, 30 mussels were collected from the waterline
19 at low tide on all sampling occasions during May 2006. Shells were opened by severance of
20 the adductor muscle followed by removal of the tissue mass. Samples of gill were taken, snap
21 frozen in liquid nitrogen and subsequently processed for species identification as described
22 below. A 3-4mm cross section was removed along a standard plane allowing for all major
23 organs and tissues (gill, mantle, kidney, digestive gland, gonad and byssus gland/foot) to be
24 incorporated into a single histological section. Excised tissues were placed into histological
25 cassettes and immediately placed into Davidson's seawater fixative. Fixation was allowed to
26 proceed for 24 h before being transferred into 70 % industrial methylated spirit (IMS).
27 Samples were transported to the Cefas, Weymouth laboratory for subsequent histological
28 processing and analysis. Samples were processed in a Vision Bio-Systems Peloris vacuum
29 infiltration processor followed by embedding in paraffin wax. Sections (3-5 μ m) were obtained
30 using a rotary microtome and subsequently stained with haematoxylin and eosin. Sections

1 were evaluated “blind” for sex and a number of health index parameters. Assessment of
2 adipogranular tissue was carried out using a qualitative index (as described by Bignell et al.,
3 2008) based on a score of 1-5 to indicate the ‘absent’, ‘present’, ‘scattered’, ‘frequent’ or
4 ‘abundant’ status of this cell type respectively. Gonadal development staging was carried out
5 as described by Seed (1976). Epithelial cell height and kidney lipofuscin were measured
6 using an index as described in Table 1. All micrographs were captured using a Nikon
7 DXM1200F digital video camera and the Lim Screen Measurement™ Lucia G image capture
8 system (Nikon, UK).

9

10 2.2. Species Identification

11 *Mytilus* populations around the South West of England are known to comprise of both *M.*
12 *edulis* and *M. galloprovincialis*, along with hybrids of both species. In order to consider the
13 effect of species on the histological health parameters recorded, individual mussels were
14 genotyped as previously described (Bignell et al., 2008). Briefly, Total DNA was extracted
15 from gill tissue using DNAzol reagent according to the manufacturers recommended protocol
16 (Invitrogen, UK). The resulting pellet was suspended in 100 µl of molecular grade water.
17 Species identification was undertaken using previously published PCR primer pairs Me15 5'-
18 CCAGTATACAAACCTGTGAAGA-3' and Me16 5'-TGTTGTCTTAATAGGTTTGTAAGA-3' for
19 the *Glu* gene (Inoue *et al.*, 1995). Species-specific products are formed with *M. edulis* and *M.*
20 *galloprovincialis* producing 180 bp and 126 bp amplification products respectively, with
21 hybrids producing both bands. PCR was performed in a 50 µl reaction volume consisting of 1x
22 GoTaq® flexi buffer containing 2.5 µl template 2.5 mM MgCl₂, 1 mM dNTPs mix, 100 pmol of
23 sense and anti-sense primers, 2.5 units of Go Taq polymerase (Promega, UK). The reaction
24 mix was overlaid with mineral oil and after pre-heating to 95°C for 5 min, subjected to 35
25 temperature cycles of: 1 min at 95°C, 1 min at 55°C and 1 min at 72°C followed by a final
26 extension step of 10 min at 72°C. Twenty µl of amplified products were electrophoresed in a 4
27 % (w/v) agarose/TAE (40 mM Tris-acetate, pH7.2, 1 mM EDTA) gel containing 1.0 µg/ml
28 ethidium bromide, and visualised under UV irradiation.

29

30 2.3 Statistical analysis

1 Cross-tables and logistic regression were used to look for associations between the presence
2 of different parasites, sampling location and the occurrence of the following dependent
3 variables: inflammation, brown cell inflammation and high gonadal status. Due to low cell
4 counts in the cross-tables and for ease of interpretation, indices for gonadal status were
5 simplified into two categories; immature and developing/ripe for the purpose of logistic
6 regression analysis. All analysis was conducted in R v.2.7.0 (R Development Core Team
7 2008). Models were built using a backward stepwise approach by first fitting the maximal
8 model and then systematically removing the least significant factors until only significant
9 factors remained.

10

11 **3. Results**

12 Thirty mussels were sampled from each site and examined for twenty seven health index
13 parameters including reproductive markers, non-specific pathologies, pathologies of the
14 digestive diverticula and infectious diseases (Table 2). Representative micrographs of
15 pathologies within these categories are presented in Figures 2 and 3.

16

17 *3.1 Reproductive markers*

18 When present, ADG cells were situated in the vesicular connective tissue. These cells stained
19 bright pink with eosin and in some cases, were granular in appearance. The median ADG rate
20 was lowest in mussels collected from Whitsand Bay (0.0) and Cremyll Ferry (0.5). Mussels
21 collected from Neal Point, Wilcove and Trebarwith Strand had a higher ADG rate in
22 comparison (2.0, 2.0 and 2.0 respectively). The median ADG rate of mussels collected from
23 Whitsand Bay and Cremyll Ferry was lower than in mussels collected from Trebarwith Strand,
24 Neal Point and Wilcove. The median gonadal stage was lowest in mussels collected from
25 Neal Point (2.0) and Wilcove (2.0). The median gonadal stage was highest in mussels
26 collected from Cremyll Ferry (3.5), Whitsand Bay (4.0) and Trebarwith Strand (3.0). Logistic
27 regression demonstrated that gonadal development was significantly better in sites
28 downstream of Neal Point (Table 3).

29

1 The prevalence of atresia (females only) was within the range of 59-100 % (Table 2) although
2 severity of the condition was not recorded. Four individual female mussels from both Cremyll
3 Ferry and Wilcove exhibited apoptosis in the germinal epithelium of the ovarian follicles. This
4 pathology has previously been confirmed by Cefas as apoptosis through a combination of
5 morphology and the terminal deoxynucleotide transferase mediated deoxy-UTP nick-end
6 labelling (TUNEL) assay.

7

8 *3.2 Non-specific pathology*

9 Three types of inflammatory lesion were observed within the numerous tissues and organs
10 examined (Table 2). Inflammatory pathologies were most prevalent in mussels collected from
11 the estuarine sampling sites. General inflammation was characterised by the presence of
12 haemocyte infiltration of the vesicular connective tissue (VCT) in and around the digestive
13 diverticula. Inflammation was observed as either focal (Fig. 1a) or diffuse lesions and was
14 most prevalent in mussels from Neal Point (56.7 %), Wilcove (50 %) and Cremyll Ferry (20
15 %). One individual exhibited extensive inflammation throughout the majority the gonadal
16 ducts. Brown cell (BC) inflammation consisting of small haemocytic aggregates (Fig.1b) was
17 observed mostly within the VCT. These aggregates could also be seen in the gills, gonadal
18 follicles, stomach wall and the epithelia of the digestive diverticula. BC inflammation within the
19 VCT was most prevalent at Cremyll Ferry (50.0 %), Wilcove (33.3) and Neal Point (26.7 %).
20 However, BC inflammation within the gonadal follicles was most prevalent at Neal Point (43.3
21 %), Wilcove (16.7 %) and Cremyll Ferry (10.0 %). BC inflammation of the gills was observed
22 at relatively low prevalence from all sites with the highest prevalence being observed in
23 mussels sampled from Wilcove (16.7 %). Pearl formations were observed at low prevalence
24 from all sampling locations (< 6.7 %). No granulocytoma were observed during this study.
25 Logistic regression showed that both the prevalence of haemocyte infiltration and BC
26 inflammation significantly decreased further downstream from Neal Point (Table 3).

27

28 Pathology of the digestive diverticula was measured in terms of reduced epithelial cell height
29 and prevalence of digestive tubule degeneration. Epithelial cell height was determined using a
30 simple index (Table 1). Mussels sampled from the Trebarwith Strand coastal site exhibited the

1 highest median epithelial cell height index thus indicating a lower epithelial thickness. Neal
2 Point, Whitsand Bay, Wilcove and Cremyll Ferry mussels showed no differences between
3 each other sharing a median epithelial cell height index of 1. Disintegration of the digestive
4 tubules was observed as either partial or complete and was often difficult to distinguish
5 between classic degeneration *per se*, or poor preservation resulting from inadequate fixation.
6 There were signs of poor tissue preservation as demonstrated by loss of cellular structure and
7 poorly defined cytoplasm and nuclei. In many cases there was loss of the apical region of
8 digestive cells situated within the digestive tubule epithelium. Extreme cases often resulted in
9 complete loss of the digestive epithelium. The highest recorded prevalence of this
10 degeneration was recorded in mussels sampled from the two coastal sampling sites of
11 Trebarwith Strand (97.0 %) and Whitsand Bay (53.0 %). The prevalence of degeneration from
12 the remaining estuarine sites was 33.3 %, 33.3 % and 13.3 % at Cremyll Ferry, Neal Point
13 and Wilcove respectively.

14

15 Lipofuscin accumulation was identified by a “yellow-brown” to “reddish-brown” colouration
16 (see Stevens and Chalk; 1996) within the kidney epithelium (Fig. 1f). Here small particles of
17 pigment could be observed of low colour intensity. Enlarged lipofuscin accumulations that
18 were more intense in colour could also be seen within some epithelial cells. This was
19 measured using a Kidney Lipofuscin (KL) index (Table 2). The median KL index at the
20 Trebarwith strand coastal site was 0 with all remaining sites showing a score of 1.

21

22 3.4 Pathogens

23 Five different pathogens were detected during this study. Mussels from Cremyll Ferry had the
24 most prevalent and widest array of pathogens. The most significant finding was of the
25 paramyxian, *Marteilia* sp., characterised by distinctive eosinophilic cell-within-cell sporocysts
26 situated within the epithelium of the digestive diverticula (Fig. 2a). This parasite was observed
27 in 31.0 % of mussels sampled from Cremyll Ferry. Hypertrophy of the digestive tubules was
28 observed in those individuals harbouring a heavy infection. *Marteilia* sp. was also observed in
29 mussels from Wilcove at a prevalence of 6.9 %. The most prevalent parasite overall was
30 *Mytilicola intestinalis* (Fig. 2b). The intestinal copepod was observed attached to the stomach

1 wall in mussels sampled from Cremyll Ferry (55.2 %), Neal Point (33.3 %), Wilcove (23.3 %)
2 and Whitsand Bay (10.0 %). *Ancistrum mytili* (Fig. 2c) and other gill ciliates were observed in
3 mussels collected from all estuarine sites with those from Cremyll Ferry exhibiting the highest
4 prevalence (26.7 %). Ciliophora-like organisms (Fig. 2d) were seen infecting the digestive
5 epithelial cells (Fig. 2e) of mussels from Cremyll Ferry (25.0 %), Whitsand Bay (16.7 %) and
6 Wilcove (3.3 %). Other intracellular pathogens of the digestive epithelium were
7 *Rickettsia/Chlamydia*-like organisms (R/CLO). Inclusion bodies were observed infecting
8 mussels from Cremyll Ferry (11.1%) and Whitsand bay (3.3 %). Of those pathogens
9 observed, *Marteilia* sp. was the only pathogen that appeared to elicit a host response as
10 demonstrated by inflammation and a reduction in ADG tissue.

11

12 3.5 Species identification

13 The species composition of the five sampling locations examined during this study differed
14 between sites and included the presence of *Mytilus edulis*, *Mytilus galloprovincialis* and
15 hybrids. All sites consisted of mixed species populations except for Cremyll Ferry, which
16 consisted solely of *M. edulis*. The species breakdown for each sampling site was as follows
17 (figures in parenthesis refer to percentage of *M. edulis*, *M. galloprovincialis* and hybrids
18 respectively): Trebarwith Strand (0, 97, 3), Whitsand Bay (37, 7, 56), Neal Point (93, 0, 7),
19 Wilcove (93.3, 3.3, 3.3), Cremyll Ferry (100, 0, 0).

20

21 4. Discussion

22 This study presents baseline histological data for twenty-seven health index parameters of
23 mussels (*Mytilus edulis*, *Mytilus galloprovincialis* and their hybrids) sampled from several
24 locations within the Tamar estuary and from two coastal sites. Histopathology lends itself well
25 to the collection of data pertinent to health status as it provides a snapshot of health at the
26 time of sampling. The data generated is helpful in providing complementary information to
27 support additional cellular and biochemical based biomarker techniques such as those used
28 in this monitoring programme (Money et al.; Shaw et al., in this issue). Hence, the use of
29 histopathology can help to dissociate markers of underlying health or condition from those
30 associated with exposure to contaminants (see Stentiford et al., 2009). Similarly, it is also

1 important to consider the effects of disease. Numerous microscopic lesions observed in
2 mussels such as inflammation and atresia, have previously been linked to a chemical
3 aetiology. However, a number of discrete pathogens, identified using histopathology can
4 exhibit characteristics that are similar in appearance to toxicopathic lesions. Consequently, it
5 is also important to consider the effects of disease and not simply the potential effects of
6 contaminants in isolation.

7

8 *4.1 Health index parameters*

9 The histological assessment of storage reserve cells (including ADG tissue) contained within
10 the mantle tissue of mussels is a marker that has been used extensively in biological effects
11 monitoring programmes using mussels. Together with the vesicular connective tissue (VCT)
12 cells, ADG cells are the primary energy reserves for fuelling gametogenesis and byssus
13 production (Lowe and Pipe, 1987; de Zwaan and Mathieu, 1992). During the warmer summer
14 months, mussels actively feed and store energy for the commencement of gametogenesis
15 during autumn and winter. As gametogenesis progresses, there is a subsequent reduction in
16 levels of glycogen and a concomitant reduction in the frequency and size of energy storage
17 cells (Seed, 1969); most notably the ADG cells. Eventually the mantle is full of ripe gametes
18 with little or no storage cells present. Following spawning (spring/early summer) this annual
19 cycle is ready to repeat once more. Depending on gametogenic condition, population location
20 or species composition, relative proportions of storage tissues to germinal cells may vary, but
21 typically follow an annual cycle. Taking these into consideration, it is generally understood
22 that deviations from a normal state, particularly with respect to a reduction in ADG tissue, can
23 be used as an indicator of contaminant exposure and physiological stress (Lowe and Pipe,
24 1987).

25

26 In this study, mussels sampled from sites further downstream in the Tamar exhibited relatively
27 more developed gonads as demonstrated by median gonadal index and logistical regression
28 analysis. Interestingly this supports biochemical and histochemical data which indicated
29 relatively higher impact at sites further upstream within the Tamar compared to those closer
30 to the mouth of the estuary (Shaw et al., in this issue). Studies have shown that exposure to

1 organic contaminants and metals result in suppressed gamete development, inhibition of
2 gonadal follicle development and/or enhancement of gamete atresia in mussels (Lowe and
3 Pipe, 1987; Kluytmans et al., 1988; Livingstone and Pipe, 1992). It is difficult to interpret the
4 differences seen in this study with respect to atresia although one might speculate that
5 chemical insult within the estuary is a contributing factor. However, with mussels from
6 Trebarwith Strand exhibiting a similar prevalence of atresia and relatively lower
7 concentrations of metals and polycyclic aromatic hydrocarbons (PAHs), it is clear that
8 numerous factors could be involved. The development of qualitative indices similar to those
9 used for other health index parameters used in this study could help to better interpret this
10 kind of data in future studies. Similarly, it is difficult to decipher the cause of apoptosis within
11 this study, especially due to the relatively low number of mussels affected (four individuals per
12 site). Elsewhere, studies in fish have noted casual links between contaminant exposure and
13 gonadal apoptosis. Marty et al (1997) showed that exposure to crude oil resulted in increased
14 gonadal apoptosis in Pink salmon (*Oncorhynchus gorbuscha*) larvae whilst Lyons et al (2004)
15 noted spermatogonial apoptosis of the viviparous blenny (*Zoarces viviparus*). Despite our
16 findings, none of the mussel populations examined appeared adversely compromised with
17 respect to their reproductive function since relatively healthy median levels of maturing
18 gonadal tissue were observed.

19

20 Conversely to gonadal index, the median ADG rate decreased downstream with mussels from
21 Cremyll Ferry and Whitsand Bay exhibiting the lowest median ADG index with fewer ADG
22 cells of decreased cytoplasmic volume present. This decreasing trend of ADG rate could be
23 indicative of the normal reproductive cycle. With that being said, mussels sampled from
24 Cremyll Ferry exhibited the widest range and highest prevalence of pathogens observed
25 within this study. The majority of the pathogens reported here do not elicit a significant host
26 response with the exception of *Marteilia* sp. Where observed, those individuals infected with
27 *Marteilia* sp. had little or no ADG tissue present. Therefore, the relatively high prevalence of
28 *Marteilia* sp. (31.0%) coupled with the pathology associated with this parasite, might also be a
29 contributing factor to the relative decrease in the median ADG rate observed at Cremyll Ferry.

1 Since its initial reporting in mussels from UK waters (Bignell et al., 2008), this is the highest
2 recorded prevalence of *Marteilia* sp. within UK waters.

3

4 Inflammatory lesions are often observed in aquatic organisms including mussels sampled
5 from stressful environments including estuaries and are commonly associated with exposure
6 to metals and organic xenobiotics (Sunila, 1986; Auffret, 1988), and pathogens (Rasmussen,
7 1986; Lowe & Moore, 1979). Following an inflammatory response, it would appear that BC
8 inflammatory aggregates migrate through the VCT to a suitable basement membrane of a
9 target organ for removal from the mussel via diapedesis. This is evident by the observation of
10 BC inflammatory aggregates within organs (and their epithelia) including the stomach,
11 intestine, digestive diverticula, gonad and kidney. Logistic regression showed no significant
12 associations between inflammatory and parasites prevalence. This could indicate (a) the
13 presence an unidentified bacterial or viral infection or (b) elevated levels of other stressors,
14 such as salinity and contaminants, at the Tamar sampling sites. Mussels sampled from the
15 sites within the Tamar not only had the highest prevalence of inflammatory lesions, but also
16 displayed a general increase in prevalence further upstream towards Neal Point (with the
17 exception of brown cell (BC) inflammation of the VCT). However, when we combined all BC
18 inflammation data together, irrespective of tissue location, logistic regression showed a
19 significant increase the closer to Neal Point. This indicated that the prevalence of the major
20 inflammatory lesions seen in this study decrease towards the mouth of the estuary.

21

22 Numerous studies have focused on the digestive diverticula as a target organ for measuring
23 abnormal changes relating to chemical exposure. These include atrophy of the digestive
24 tubules, an increase in the basophil cell complement, increased lipid accumulation within
25 digestive epithelial cells as well as measurements of lysosomal stability (Lowe et al., 1981;
26 Lowe & Clarke, 1989; Lowe & Pipe, 1994). In this study we observed degeneration of the
27 digestive tubules in many of the mussels examined. Degeneration of the digestive tubules has
28 previously been linked to toxicity (Lowe and Clarke, 1989, Rasmussen, 1980), however it is
29 interesting that those sampled from Trebarwith Strand exhibited the highest prevalence of
30 tubule disintegration (Table 2). Dimitriadis and Koukouzika (2003) previously showed that

1 disintegration of the digestive tubules, including poorly defined apical cell line and cytoplasmic
2 content within the tubule lumen, can occur as little as 2 hours following removal from
3 seawater. Although care was taken to standardise sampling of animals from all field sites, it is
4 possible that the increased distance of Trebarwith Strand from the laboratory where
5 dissection and fixation of specimens occurred may have contributed to the phenomenon.

6
7 Previous studies have demonstrated lipofuscin accumulation within pathologically altered
8 lysosomes of epithelial cells of the digestive diverticula following exposure to xenobiotics
9 (Moore et al., 1987; Lowe & Clark, 1989). We investigated lipofuscin accumulation within
10 lysosomes situated in the kidney epithelium, which were observed and recorded in using a
11 qualitative index (Table.1). The kidney is an important organ for the excretion of
12 unmetabolised metals and is rich in tertiary lysosomes containing metal-binding lipofuscin
13 (Livingstone & Pipe, 1992). These lysosomes are subsequently excreted in the urine following
14 exocytosis. It is commonplace to observe relatively low levels of lipofuscin-containing
15 lysosomes within the kidney epithelium. Although no gradient response was observed
16 between the estuarine sampling sites, those within the Tamar and from Whitsand Bay shared
17 a median kidney lipofuscin (KL) index of '1' compared to mussels sampled from Trebarwith
18 Strand that had a median KL index of '0'. This result supports those observations of Shaw et
19 al. (in this issue) who concluded that levels of metals in mussels within the Tamar estuary and
20 at Whitsand Bay were an order of magnitude higher than baseline levels for the UK.
21 Furthermore, levels of metals at Trebarwith Strand were between one and two orders of
22 magnitude lower than sampling sites situated in the Tamar.

23 24 *4.2 Effect of species*

25 Few studies have investigated the effects of species on measurements such as parasitic
26 infection, metal accumulation, contaminant related protein expression, and pathology (Lobel
27 et al., 1990; Coustau et al., 1991; López et al., 2002; Bignell et al., 2008). In this study
28 genotyping was carried out on individual mussels in order to (a) determine the species
29 distribution of mussels sampled from the estuarine and coastal sampling sites and (b) take
30 into consideration any potential data variation that may be attributed to species differences. It

1 is noteworthy that the majority of mussels sampled in this study were *M. edulis*. However,
2 97% of mussels sampled from the coastal site of Trebarwith Strand were *M. galloprovincialis*.
3 This confirms the work of Skibinski et al. (1982) and Hilbish et al. (2002) that extensively
4 characterised the south western hybrid zone in the UK. Despite species being determined for
5 all individual mussels, no species differences regarding histopathology could be investigated
6 due to insufficient numbers of individual “species” at each of the sampling sites. Although no
7 differences were detected here, studies have previously shown that the reproductive cycle
8 and pathology may differ significantly between different mussel species collected from the
9 same location and at the same time (Bignell et al., 2008; Secor et al., 2001). Whether the
10 hybrid complex of mussel species collected as part of this study can affect biomarker
11 response or contaminant bioaccumulation remains to be shown and as such could be a target
12 of future studies where mussels from hybrid populations are to be used to assess
13 environmental status.

14

15 **5. Conclusions**

16 Histopathology has been used extensively for the investigation of health in aquatic organisms
17 and is complementary to other techniques used in biological effects programmes. The aim of
18 this study was to undertake a histopathology survey of mussels from the River Tamar,
19 surrounding area and at an additional coastal site, in the context of biological effects
20 monitoring. Several of the histological health index parameters used here confirm the findings
21 of Shaw et al. (this issue), demonstrating that sites situated in the upper part of the Tamar
22 estuary were relatively more impacted when compared with those closer to the coast. Future
23 studies incorporating mussel histopathology should at least consider the use of these
24 parameters including gonadal and adipogranular index, inflammatory lesions and kidney
25 lipofuscin (KL). It is also recommended that other histological criteria such as those measured
26 in relation to the digestive diverticula, are also included wherever possible if target organs of
27 interest are available.

28

29 During this study, we experienced an artefact (degeneration of the digestive diverticula) that
30 was potentially linked to air exposure or inadequate tissue preservation. Nonetheless, with

1 mussels becoming commonplace in programmes that monitor the biological effects of
2 contaminants, it is timely to consider quality assurance in the manner in which mussels are
3 sampled for mussel histopathology and other biological effects. Particularly in national
4 programmes that encompass sites over a large geographical area. Integrated programmes
5 that measure the biological effects of contaminants are becoming more commonplace
6 therefore this will surely benefit all those techniques that are utilised. Similar guidelines are
7 already in place for estuarine and offshore monitoring programmes utilising flatfish (Feist et
8 al., 2004). In this respect, International Council for Exploration of the Seas (ICES) guidelines
9 for conducting histopathology on mussels have been initiated through the ICES Working
10 Group on the Biological Effects of Contaminants (WGBEC). It is also essential that grading
11 criteria for histological health parameters are developed and validated for use in
12 environmental monitoring programmes, Their development, along with appropriate AQC
13 protocols will strengthen the use of mussels as biomonitoring organisms and facilitate their
14 inclusion in International programmes, classifying the ecological health status of aquatic
15 ecosystems, such as the Water Framework Directive and the new EU Marine Strategy
16 Framework Directive (Haggart *et al.*, 2008; Thain et al., 2008).

17

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19

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23

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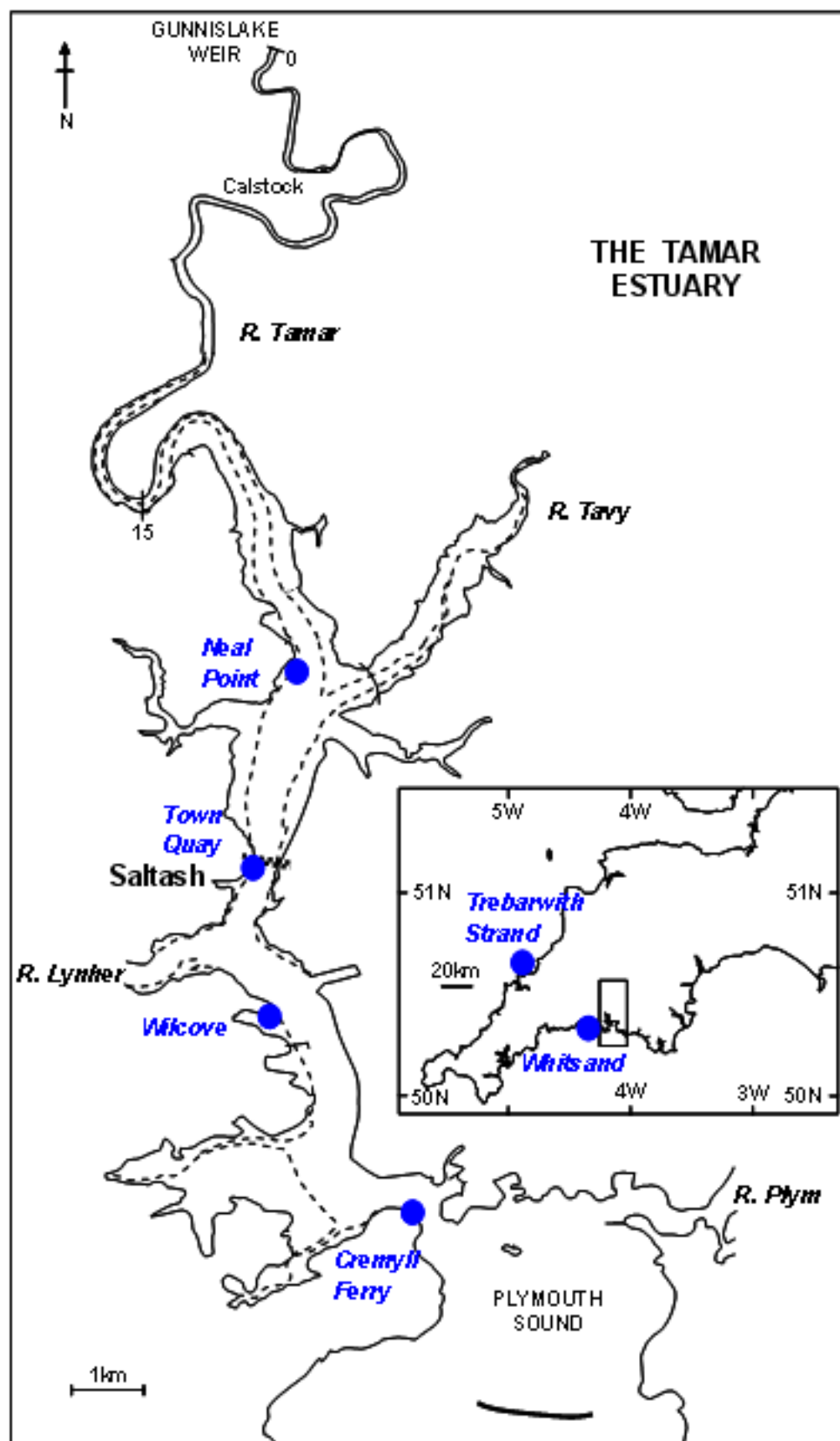
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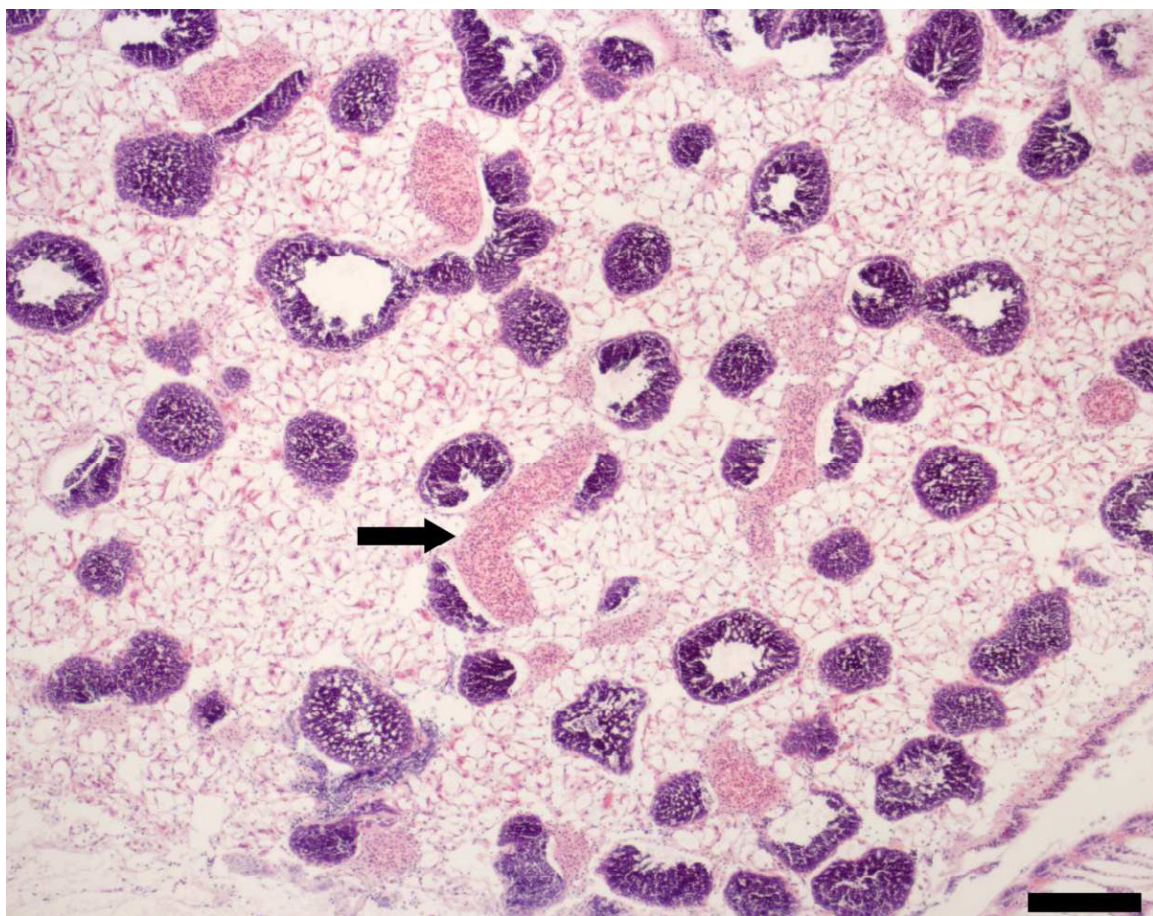
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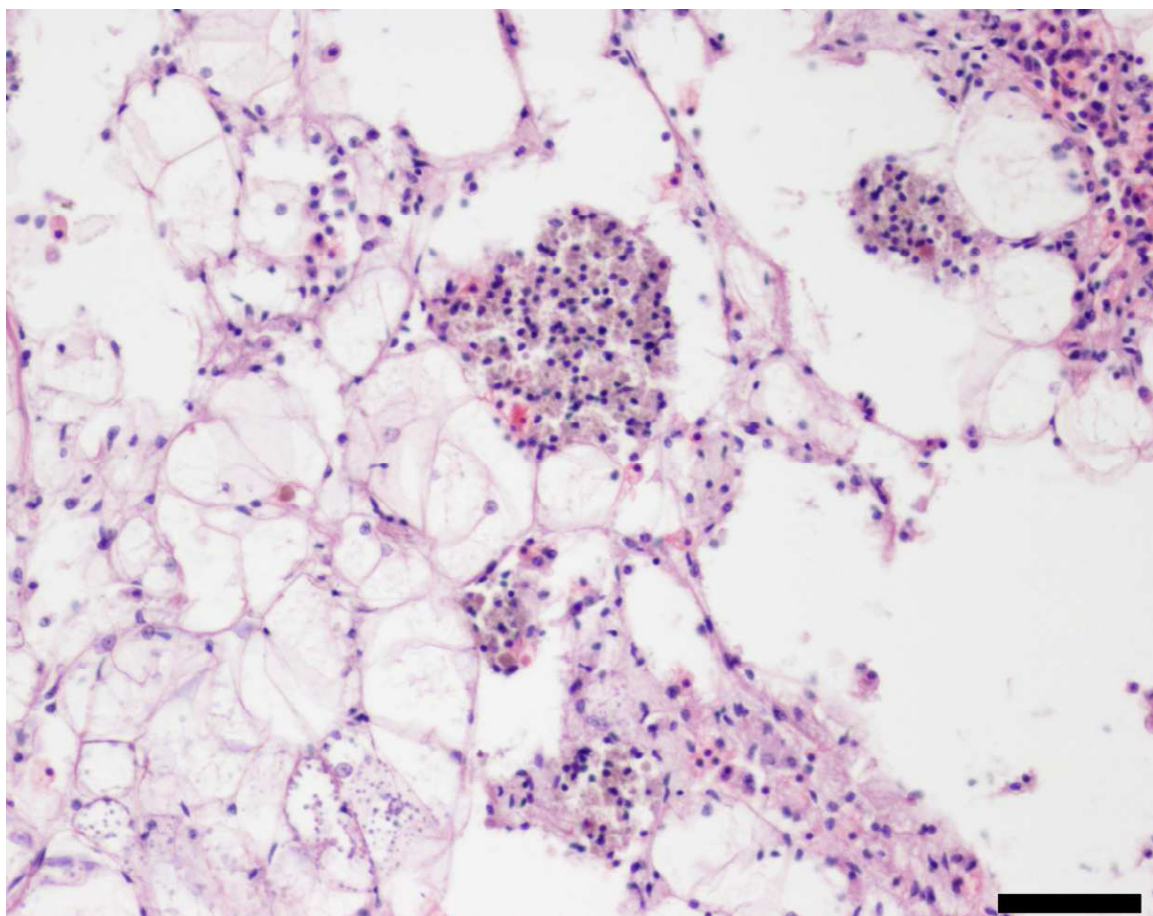
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- Reproductive condition and inflammation showed contamination gradient response.
- Estuarine locations exhibited increased prevalence of inflammatory lesions.
- Relatively higher occurrence of kidney lipofuscin from estuarine sites.
- *Marteilia* sp. observed in 31% of mussels sampled from Cremyll Ferry site.

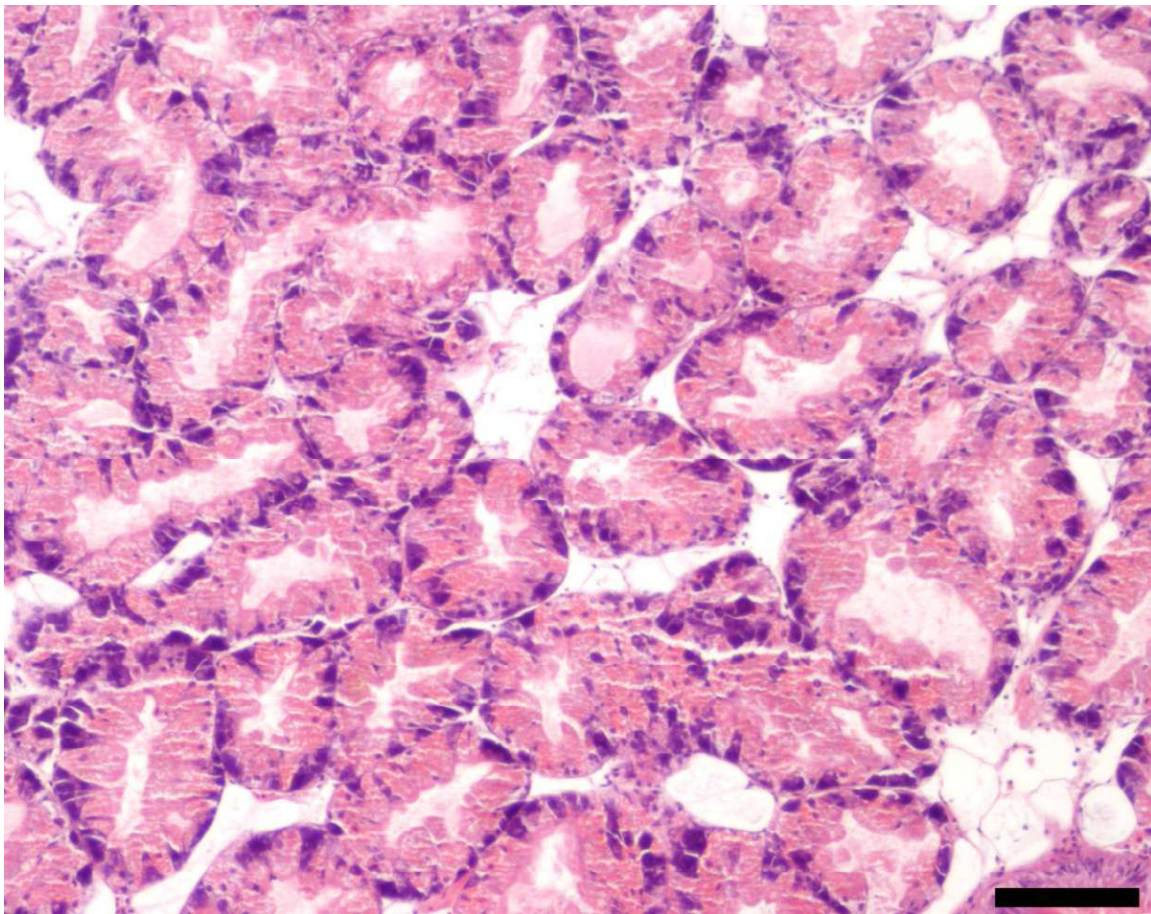




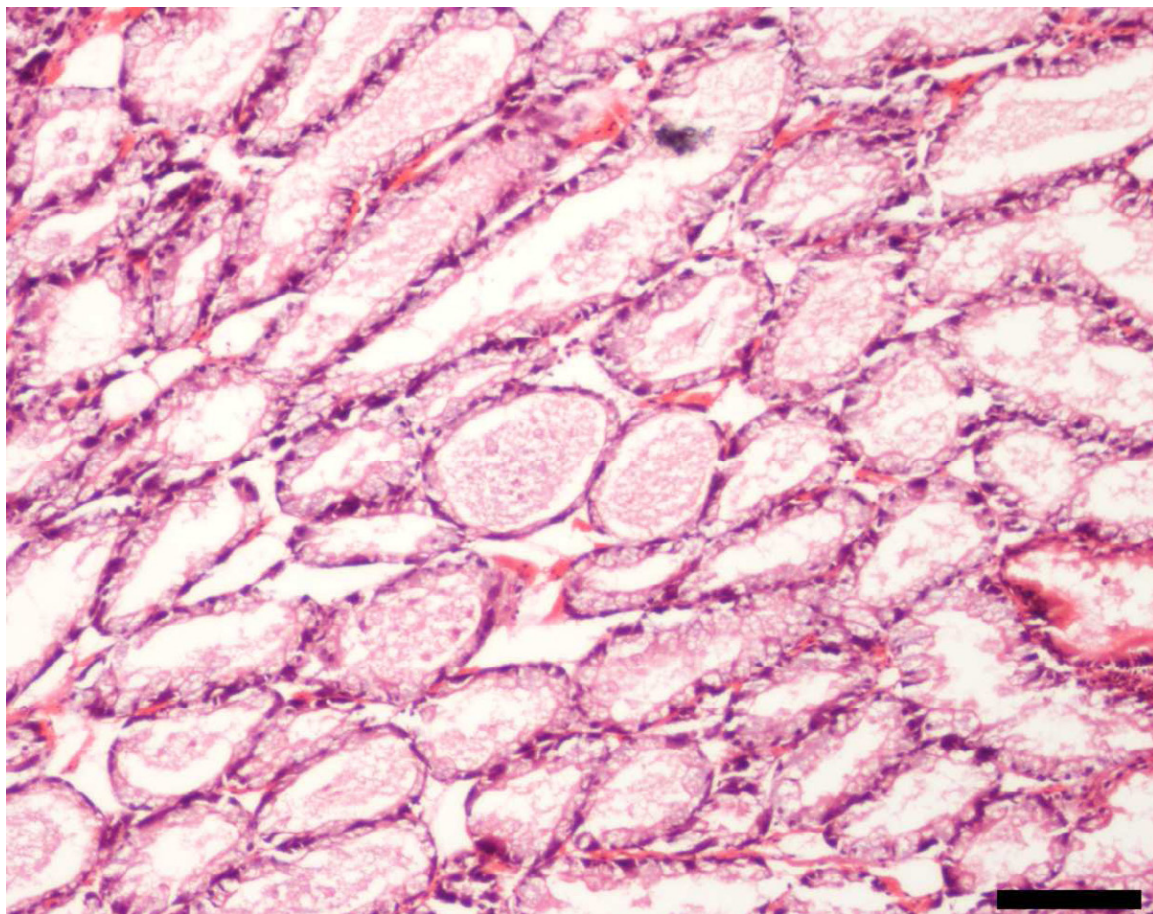
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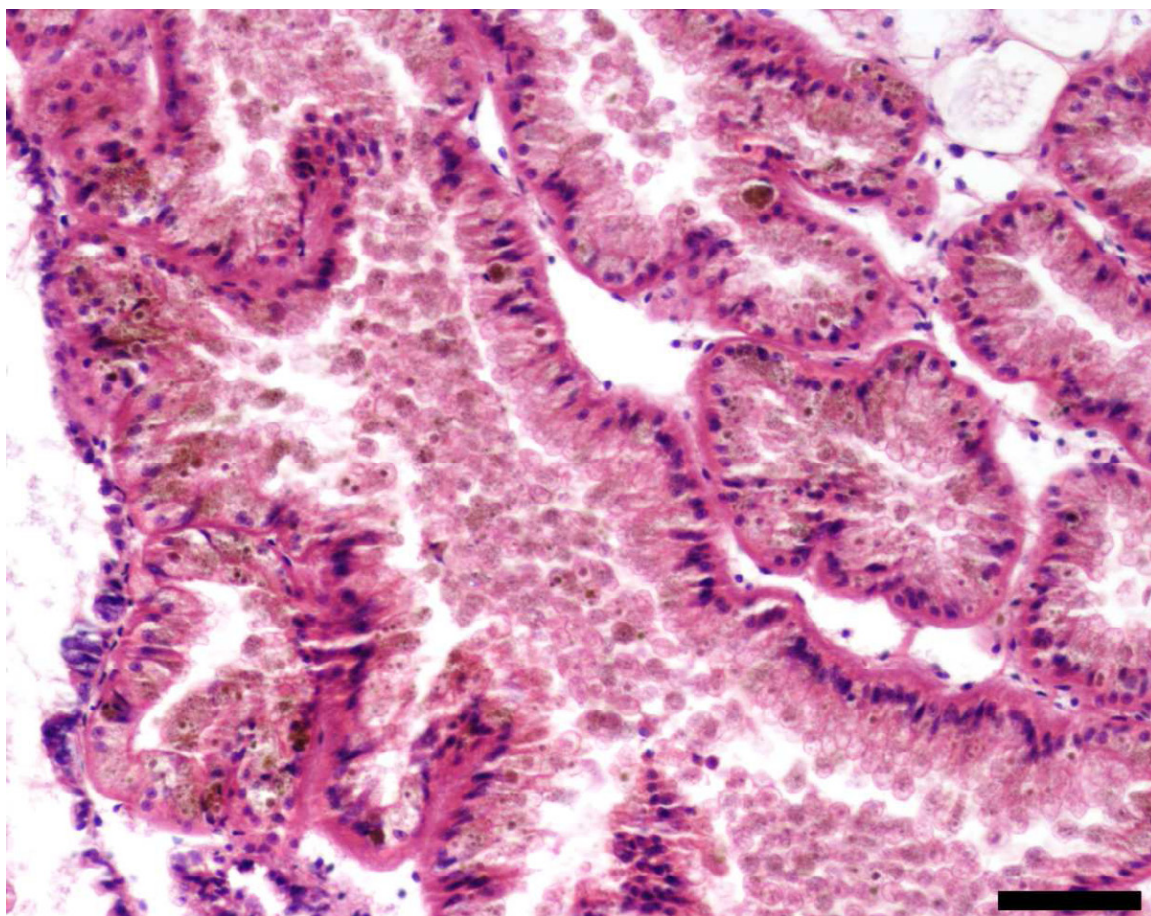
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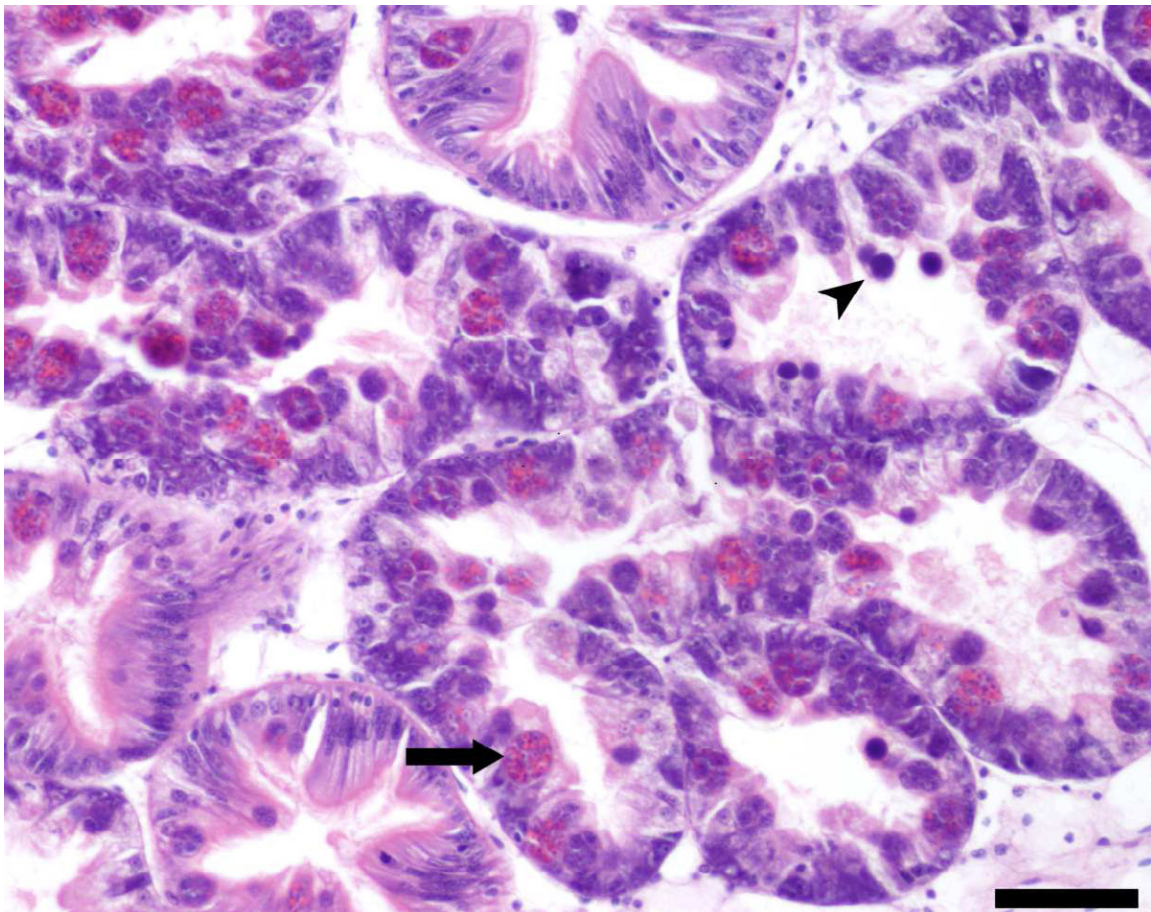
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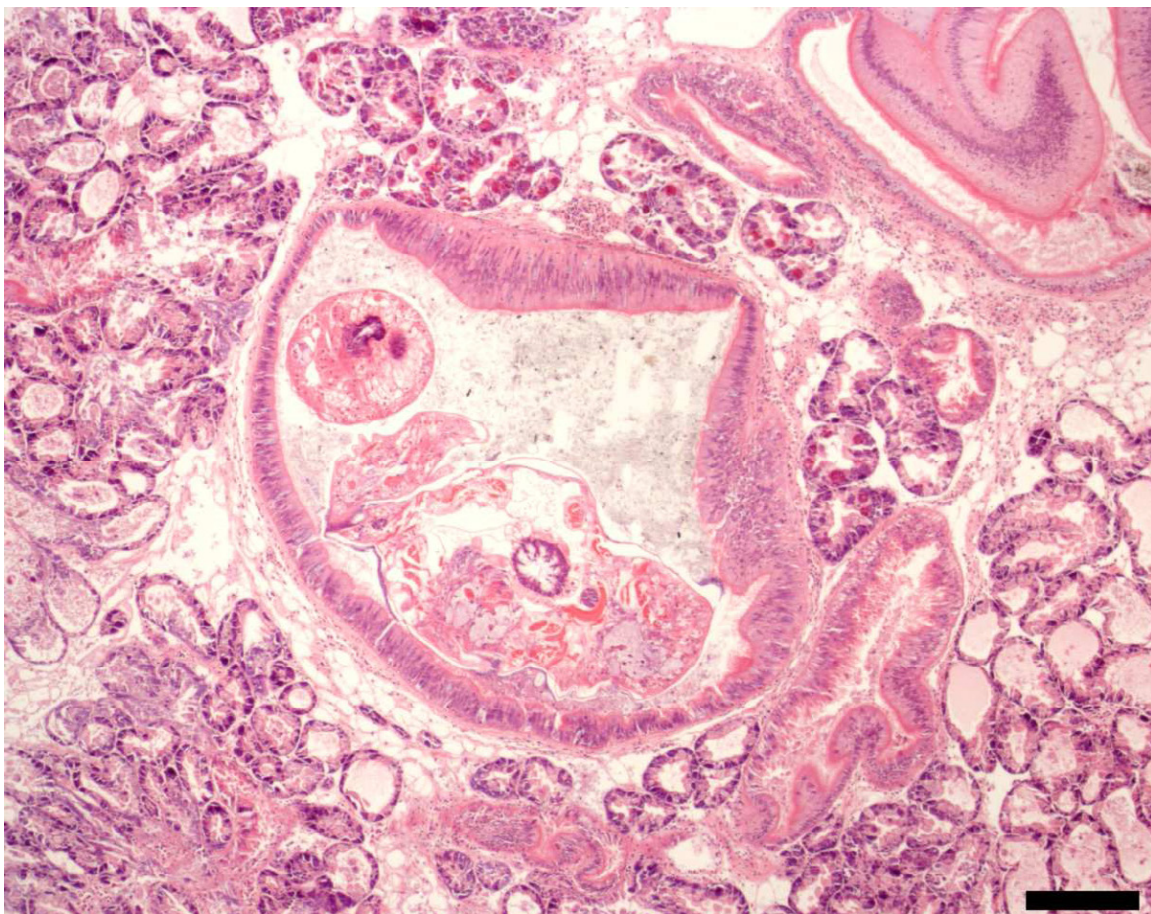
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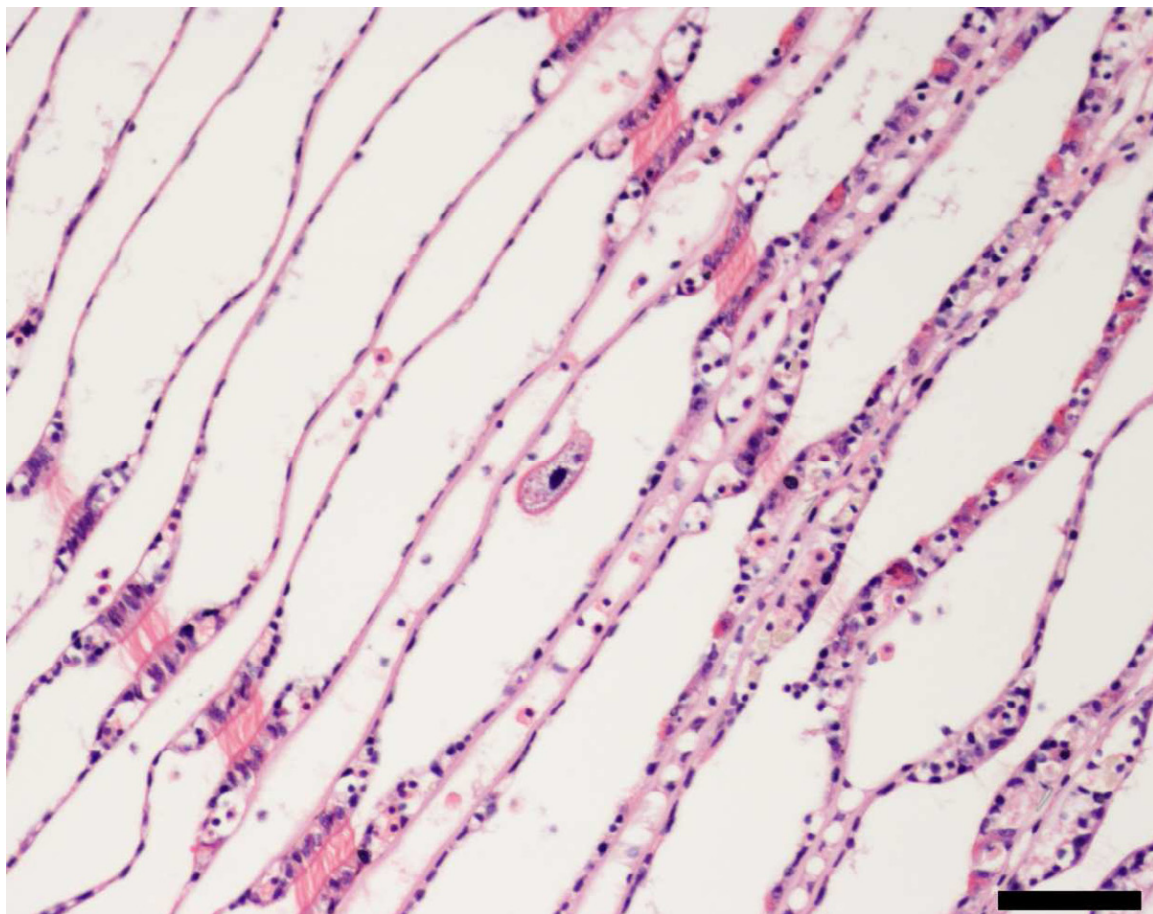
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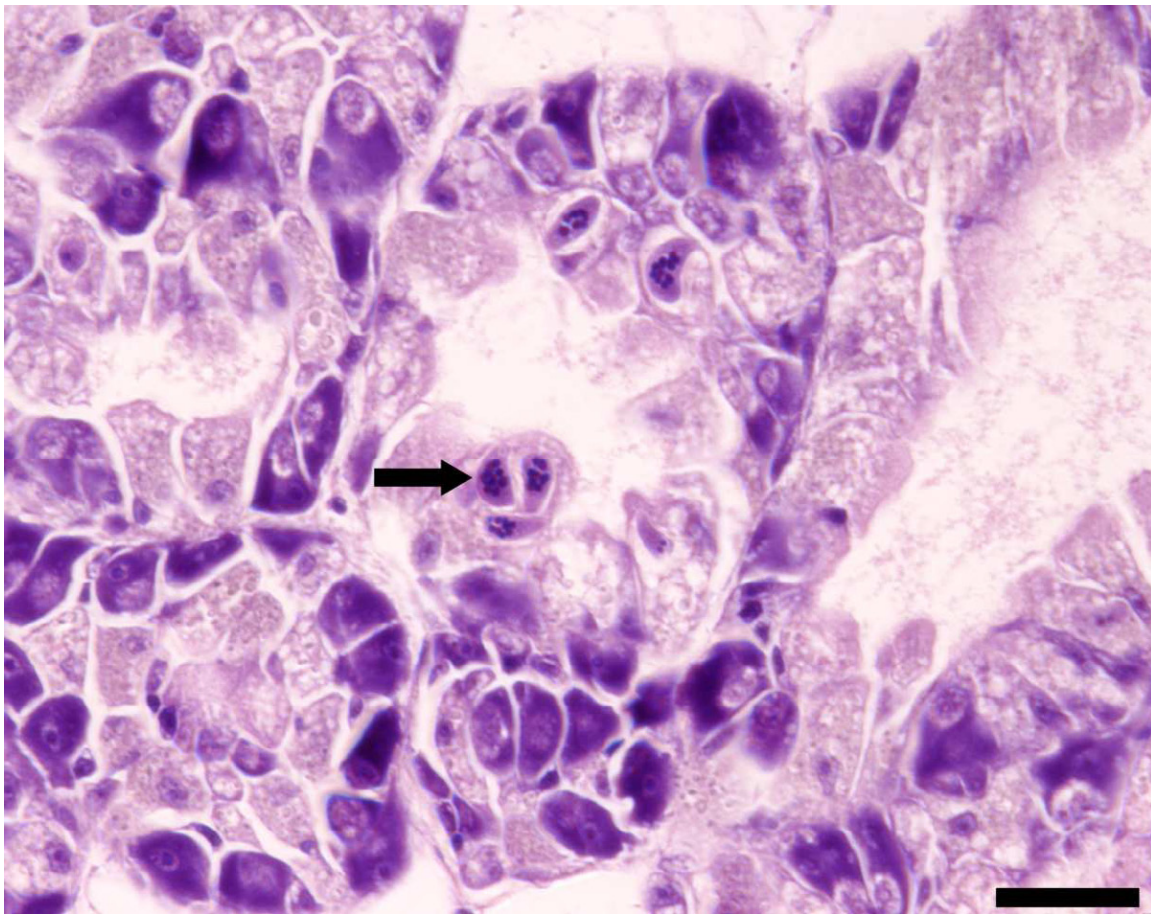
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Figure legend

Figure 1: Map displaying the sampling locations within the Tamar Estuary and Trebarwith Strand.

Figure 2: Histopathology of mussels.

- (a) Multiple inflammatory foci (arrow) within the vesicular connective tissue (VCT) of individual mussel from Wilcove. Scale bar= 200 μm
- (b) Small foci of brown cell inflammation situated within the VCT. Scale bar= 50 μm
- (c) Representative micrograph of digestive tubules in the absorptive phase. Scale bar= 100 μm
- (d) Digestive tubules (reconstituting phase) of mussel from Trebarwith Strand. Note the reduced epithelial cell height of the digestive tubules. Scale bar= 100 μm
- (e) Lipofuscin accumulation within lysosomes of kidney epithelial cells. Note the presence of small particular and larger aggregations of lipofuscin accumulation. Scale bar= 50 μm

Figure 3: Histopathology of mussels.

- (a) Marteiliosis. Eosinophilic “cell-within-cell” sporocysts clearly identified within the epithelium of the digestive diverticula (arrow). Note co-infection with Rickettsia/Chlamydia-like organisms (arrowhead). Scale bar= 50 μm
- (b) *Mytilicola intestinalis* located within the stomach. These copepods attach to the stomach wall epithelium using hook attachments. Scale bar= 200 μm
- (c) *Ancistrum mytili* attached to gill lamellae. Note the presence of pear-shaped body, single large macronucleus and micronuclei. Scale bar= 50 μm
- (d) Ciliophora-like organisms infecting the digestive epithelial cells. Note the presence of multiple parasites within one digestive cell (arrow). Scale bar= 25 μm .

Table 1: Grading indices used for the assessment of Kidney Lipofuscin (KL) and Digestive tubule epithelial cell height index.

KIDNEY LIPOFUSCIN

Stage 0 Absent
No lipofuscin present within kidney epithelial cells

Stage 1 Present
Initial lipofuscin appears faint within epithelial cells

Stage 2 Intermediate
A marked increase in the intensity of lipofuscin. $\leq 70\%$ of cell affected. Most cells display these staining characteristics.

Stage 3 Abundant
All cells contain $\geq 70\%$ of intense lipofuscin.

DIGESTIVE TUBULE EPITHELIAL CELL HEIGHT

Stage 0 Normal
Tubules nearly occluded, slight atrophy in few tubules

Stage 1 Slight
Slight atrophy to 50% normal tubule thickness

Stage 2 Intermediate
One half-tubule thickness to significant atrophy. 50-75% normal tubule thickness

Stage 3 Severe
Extremely thin, severe, most tubules affected. 75%+ normal tubule thickness

	Index Range	Neal Point	Wilcove	Cremyll Ferry	Whitsand Bay	Trebarwith Strand
Gonadal index* ^(v)	0-5	2	2	3.5	4	3
Adipogranular (ADG) index* ^(vi)	0-5	2	2	0.5	0	2
Epithelial cell height index* ⁽ⁱ⁾	0-3	1	1	1	1	2
Kidney lipofuscin index* ^(vi)	0-3	1	1	1	1	0
*Median index						
Apoptosis ^{(v) ♀}		0.0	25.0	25.0	0.0	0.0
Atresia ^{(v) ♀}		59.0	75.0	63.3	100.0	81.0
Intersex/hermaphrodite ^(v)		0.0	0.0	0.0	0.0	0.0
Inflammation ^(vi)		56.7	50.0	20.0	3.3	16.7
Granulocytoma ^(vi)		0.0	0.0	0.0	0.0	0.0
Brown cell (BC) inflammation ^(v)		43.3	16.7	10.0	0.0	3.3
Brown cell (BC) inflammation ^(vi)		6.7	16.7	6.7	3.3	0.0
Brown cell (BC) inflammation ^(vi)		26.7	33.3	50.0	16.7	0.0
Pearl formations ^(vi)		0.0	3.3	6.7	3.3	3.3
Lysosomes ⁽ⁱ⁾		56.7	40.0	51.9	33.3	0.0
Eosinophilic bodies ⁽ⁱ⁾		96.7	93.3	44.4	26.7	0.0
Degeneration ⁽ⁱ⁾		33.3	13.3	33.3	53.3	96.7
Rickettsia/Chlamydia-like organisms ⁽ⁱ⁾		0.0	0.0	11.1	3.3	0.0
Rickettsia/Chlamydia-like organisms ⁽ⁱ⁾		0.0	0.0	0.0	0.0	0.0
Ancistrum mytili / other ciliates ⁽ⁱ⁾		6.7	20.0	26.7	0.0	0.0
Gregarine ⁽ⁱ⁾		0.0	0.0	0.0	3.3	0.0
Digenean metaceccarial stages ⁽ⁱ⁾		0.0	0.0	6.7	6.7	0.0
<i>Marteilia</i> sp. ^(i, iv)		0.0	6.9	31.0	0.0	0.0
<i>Mytilicola intestinalis</i> ^(iv)		33.3	23.3	55.2	10.0	0.0
<i>Steinhausia mytilovum</i> ^{(v) ♀}		0.0	0.0	0.0	0.0	0.0
Copepod ⁽ⁱ⁾		0.0	0.0	0.0	0.0	0.0
Haplosporidian ^(i, iii)		0.0	0.0	0.0	0.0	0.0
Ciliophora-like organism ⁽ⁱ⁾		0.0	3.3	25.0	16.7	0.0
Bucephalis ^(vi, v)		0.0	0.0	0.0	0.0	0.0
Haemocytic neoplasia ^(i, iii)		0.0	0.0	0.0	0.0	0.0
Neoplasia ^(vii)		0.0	0.0	0.0	0.0	0.0

i= digestive diverticula, ii= gill, iii= vesicular connective tissue, iv= stomach, v= gonad, vi= kidney, vii= various tissues
 ♀ Prevalence shown as percentage of females only

Table 2: Data for the twenty-seven health index parameters of *Mytilus* sp.. All data presented as percentage prevalence except where specified.

Table 3: Logistic regression results showing the relationship between the different study sites and the presence of histopathological indicators. Sites compared to the most upstream site: Neal Point.

Gonad development

Coefficients:

	Estimate	Std. Error	z value	Pr(> z)
(Intercept)	-0.6931	0.387	-1.790	0.074
Wilcove	-0.4964	0.580	-0.856	0.392
Cremyll Ferry	1.7047	0.566	3.011	0.003
Whitsand Bay	4.0604	1.088	3.731	0.000
Trebarwith Strand	2.0794	0.599	3.474	0.001

Inflammation (haemocyte infiltration)

(Intercept)	0.268	0.368	0.728	0.467
Wilcove	-0.268	0.519	-0.517	0.605
Cremyll Ferry	-1.655	0.587	-2.821	0.005
Whitsand Bay	-3.636	1.082	-3.361	0.001
Trebarwith Strand	-1.878	0.613	-3.063	0.002

Brown cell (BC) inflammation

(Intercept)	0.406	0.373	1.088	0.277
Wilcove	-0.539	0.522	-1.032	0.302
Cremyll Ferry	-0.272	0.522	-0.521	0.603
Whitsand Bay	-1.792	0.589	-3.041	0.002
Trebarwith Strand	-3.772	1.083	-3.483	0.000

DOWNSTREAM

