



Research Article

Histological Examination of *Perna canaliculus* Mussels during a Summer Mortality Event in New Zealand

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The New Zealand Greenshell™ mussel (*Perna canaliculus*) is endemic to New Zealand and contributes to the success of the country's aquaculture industry. However, summer mortality and potential disease outbreak events are having an increasing effect on the growth of this industry. The cause of these mortalities remains unknown, and histopathological studies of the pathogen and parasites in mussels are still incomplete. In the present study, a histological approach was used to identify pathogens and parasites, as well as immunological tissue responses in unhealthy- and healthy-looking *P. canaliculus* during a summer mortality event in 2018. A highly significant association between health conditions and the presence of *Perkinsus olseni* in mussels was observed. A higher prevalence of *P. olseni*, Apicomplexan-X (APX), and bacterial (rods and cocci) infections were noted in the unhealthy-looking mussels than in the healthy-looking mussels. In an assessment of stains, hematoxylin and eosin (H&E) staining appeared to be the best method for general pathological and anatomical characterization, while Giemsa provided the clearest visual definition of bacteria. In this aspect, it was comparable to Ziehl–Neelsen (ZN) in apparent sensitivity. Although Gram and ZN staining revealed bacterial cells marginally better than with H&E, their differential staining could not be assessed as no Gram-positive or acid-fast bacteria were seen, and no mussel-positive controls were available for comparison. This study also provides an illustrated guide to some significant mussel health indicators.

1. Introduction

The New Zealand Greenshell™ mussel (*Perna canaliculus*) is a significant aquaculture species in New Zealand [1, 2]. The country produced 99,700 tonnes of *P. canaliculus* in 2017, which accounted for 85.6% of total aquaculture production (reviewed by Nguyen [2]). Compared to other shellfish species, both farmed and wild *P. canaliculus* mussels experience fewer health issues [3]. However, the presence of several pathogens and parasites has been identified in *P. canaliculus*, including *Vibrio* spp. [4], digestive epithelial virosis [5], *Microsporidium rapuae*, and *Bucephalus* spp. [3, 6], *Tergestia agnostomi* [7], rickettsiae and apicomplexan parasite X (APX) [6, 8, 9], and *Perkinsus olseni* [6, 10, 11]. In addition, challenge experiments of *P. canaliculus* with *Vibrio splendidus* and a *Vibrio coralliilyticus/neptunius*-like isolate and *Vibrio* sp.

DOI isolated from *P. canaliculus* have demonstrated the pathogenicity of these two species to both larval and adult mussel stages [4, 12, 13].

During the last decades, high/mass mortality events have become a frequent phenomenon in shellfish worldwide [1, 14–17]. Such events often occur during summer months when the temperatures are elevated and are termed summer mortalities. Pathogens, including viruses (such as ostreid herpes virus 1) [14, 18], bacteria (such as *Vibrio* spp.) [19], and parasites like protozoans [20], have been detected in association with these events. In recent summers, unexplained mortalities of *Perna canaliculus* have been reported in many farms throughout New Zealand [21], and the nature of these events is not well understood. Pathogen identification during these events is lacking, and there is an urgent need to investigate the causative agents and complex

host–pathogen–environment interactions that occur during these mortality events, as high mortalities of this endemic mussel species in both wild and farm stocks have become a frequent occurrence [10].

Shellfish summer mortality events often occur due to the complex interactions between pathogens, host physiological state, and environmental factors. These events are typically associated with an increase in temperature [14, 22]. When the temperature is high, some parasites are likely to increase their transmission between hosts [23, 24, 25]. Bacterial proliferation in warm water and accumulation in shellfish tissues can cause stress, diseases, and mortality [26, 27]. Histology is a common technique used for identifying the effects of disease and parasite infection in shellfish [28–30]. It also provides dependable proof of cell or tissue damage and inflammatory responses [31]. Histology is a cost-effective and reliable technique for identifying and localizing bacteria [32]. The process of histology involves five key steps, including fixation, processing, embedding, sectioning, and staining [33]. Within this process, the staining step not only enhances tissue contrast but also highlights important features of the tissue. [34]. Hematoxylin and eosin (H&E) staining can be used to prepare sample tissues for better visualization of structures, cell types, or pathogens (e.g., bacteria) during microscopic observation [34, 35]. H&E, Gram, and Giemsa staining are three common staining techniques used in histology. Among these, H&E, which uses basophilic and acidophilic stains, is perhaps the most common staining technique used in routine histologic examinations [36]. However, although H&E can detect bacteria [32], it cannot distinguish variations in biochemical response, such as seen in Gram staining and other methods. Consequently, individual microorganisms are not effectively related to H&E staining alone [37]. To this end, Gram staining is the most widely recognized diagnostic staining method utilized in clinical settings and in research centers to separate Gram-positive and Gram-negative microbes in various tissues between Gram-positive and Gram-negative bacteria in various tissues [37, 38]. Another common staining technique used in histology is Giemsa staining, which is sensitive, inexpensive, easy to perform, and reproducible, making it one of the most used methods worldwide [39]. In addition, the diagnosis of mycobacterial infection relies upon the Ziehl–Neelsen (ZN) stain, which detects mycobacteria due to their characteristic acid-fast cell wall synthesis and structure [40]. Therefore, the combination of multiple histological stains could provide more accuracy of pathogen identification and tissue inflammation.

During a summer mortality event in April 2018, a detailed histopathological examination was carried out on cultivated New Zealand Greenshell™ mussels (*P. canaliculus*) to recognize any potential pathogens and parasites. The purpose of this examination was to evaluate the health condition of the mussels during the event. Apart from bacterial detection, the present contribution aimed to compare different staining methods for shellfish histopathological assessment.

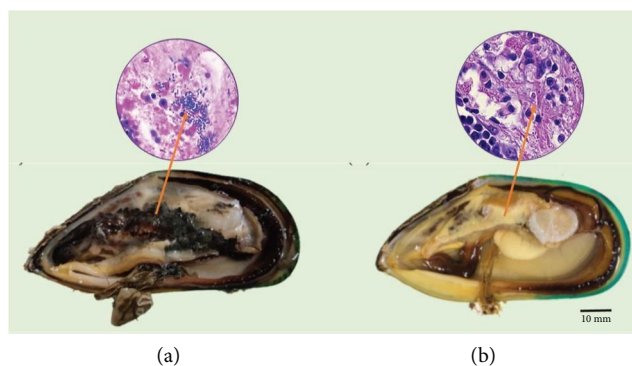


FIGURE 1: Internal organs of unhealthy (a) and healthy (b) *P. canaliculus* mussel collected from Kaiaua mussel farm in New Zealand during a high mortality event in 2018. Histology slide staining with H&E showed that rod shaped bacteria were numerous in unhealthy mussel tissues, but rare in healthy mussel tissues. Scale bars = 10 mm.

2. Materials and Methods

2.1. Sample Collection. Fifteen adult mussels (*P. canaliculus*) were gathered from a mussel farm in Kaiaua (Whakatipu, New Zealand: 37°02' 51.2' S 175°18'56.1' E) in April 2018 (average sea surface temperature 20.3°C) when the farm experienced a high mortality event. All the mussels sampled were grown-ups of 24 months old (weight 59.96 ± 8.56 g; shell length 9.4 ± 0.48 cm), gathered from around 5 m beneath the water surface. There were two types of mussel lines: healthy and unhealthy. The healthy lines had very few dead mussels, while the unhealthy lines had an estimated mortality rate of 60%–80% (deemed affected by summer mortality) based on observations and farm records (Figure 1). The sampling for this project was opportunistic as part of a larger response project to sample mussels quickly for biochemical and omics analyses aimed to capture health signatures as mussels were dying. The two mussel groups (seven unhealthy and eight healthy) were kept in two isolated polystyrene cooler boxes (Esky) with seawater taken from the farm and afterward transported (3-hr transport time) to the Aquaculture Laboratory at the Auckland University of Technology (Auckland, New Zealand).

Upon arrival at the laboratory, samples were processed. Mussels were examined for their behavioral responses, such as shell closing or gaping, when gently manipulated. The group of mussels that had experienced high mortality displayed a much slower response time in terms of shell closure, as compared to the other group. It is worth mentioning that all the mussels examined were found to be alive, as determined by their shell closure.

2.2. Histology. After measuring the weight and shell length, all 15 specimens (seven unhealthy and eight healthy) were opened to remove the soft parts (including gills, mantle, muscle, digestive tubules, digestive epithelium, and gonads). The soft parts were then cut into 2–5 mm thick slices following the method described by Howard et al. [41]. The slices

were processed using standard histological techniques as outlined by the OIE [42]. The tissue slices were placed in histological cassettes and fixed in 4% formalin (in filtered seawater) for 48 hr. Then, they were transferred to 70% ethanol for storage. The specimens were dehydrated in a series of ascending concentrations of ethanol with two changes of xylene and then embedded in paraffin wax. Using a microtome, sections of 5 μm were obtained. Slides with adhering tissue sections were dewaxed in xylene and afterward rehydrated through a descending series of ethanol concentrations followed by distilled water. Slides were stained with regressive Mayer's H&E stains, rinsed with deionized water, and afterward taken through an ascending series of ethanol concentrations. From that point forward, slides were rinsed in two changes of xylene. Finally, DPX mountant was utilized to seal glass coverslips over the sections. Similar processing followed by Gram, ZN, or Giemsa staining provided comparison preparations to investigate the best stain for detecting and identifying bacteria in *P. canaliculus*.

2.3. Microscopic Observations. Tissues that were examined included gill epithelium and blood spaces, gonad, mantle, palp, muscle, and nervous tissue, gut, digestive tubule connective tissue, epithelium, and lumen.

Prepared histological slides stained with H&E were examined under the microscope (Leica DM2000 microscope, Manufacturer-Leica Microsystems, Germany) using 4x, 10x, and 40x objectives (the 4x is bright field, and the 10x and 40x objectives are phase contrast) for identifying parasites (*Perkinsus olseni* and APX) and pathogens, as well as, for assessing immunological tissue responses. A 100x objective (oil) (phase contrast) was used for closer examination as required. Prepared histological slides staining with H&E, Gram, ZN, and Giemsa were examined under a microscope (Leica DM2000). To detect bacteria (bacilli and cocci) in different tissues of mussels, 4x, 10x, and 40x objectives were used (the 4x is bright field and the 10x and 40x objectives are phase contrast). Images were taken using a Leica ICC50 HD Microscope Camera (Manufacturer-Leica Microsystems, Germany) on the microscope (Leica DM2000) with 40x and 100x objectives (phase contrast).

During the examination, each tissue was thoroughly checked to determine the number of parasites (*P. olseni* and APX). This information was then used to calculate the parasite prevalence per individual by visually examining a single histological section of each case at 40x and 100x magnification. Prevalences were calculated using the formula established by Bush et al. [43]. Additionally, the presence or absence of bacteria (bacilli and cocci) in the various tissues was recorded as either present (1) or absent (0).

Ceroid accumulation and hemocytosis were noted as inflammatory responses. To grade the concentration of ceroid material, a semi-quantitative scale [44] was used, where Grade 1 indicates a light concentration, Grade 2 indicates a moderate concentration, and Grade 3 indicates a heavy concentration. Haemocytosis was noted as the presence or absence of an abnormally elevated number of hemocytes in a tissue area. All samples had some presence of hemocytes,

which is the normal condition, while a few or medium numbers of hemocytes in a tissue area indicated their absence.

2.4. Statistical Analyses. Statistical analyses were conducted using IBM® SPSS® Statistics software (version 23) and Pearson's *chi*-square test. Pearson's *chi*-square test statistics and the associated *p*-values were applied used to examine the relationship between health conditions and parasite infection, with statistical significance at $p < 0.05$.

3. Results

3.1. Healthy vs. Unhealthy Mussels. Upon examination, clear differences were observed between healthy and unhealthy mussels. Unhealthy mussels had extensively damaged tissues, with mucus present on external organs that appeared paler than those of healthy mussels (Figures 1(a) and 1(b)). In addition, histology slide staining with H&E from unhealthy mussels showing numerous *Vibrio* bacteria (rod and cocci), and healthy mussels showing no or rare bacteria (rod-shaped bacteria present in three mussel samples) (Figures 1(a) and 1(b), Table 1).

3.2. Infection with *P. olseni*. Trophozoites of *P. olseni* were observed in various tissues of mussels, which include connective tissues surrounding the digestive tract epithelium, digestive tubules, gonads, gills, mantle, and adductor muscle. These trophozoites are spherical cells with a diameter of 3–5 μm , and they exhibit a “signet ring” appearance due to their peripheral nucleus and a large, eccentric vacuole occupying most of their cytoplasm (Figures 2(a) and 2(b)) [10, 45].

3.3. Infection with APX. Observations were made of APX zoites present in the connective tissue that surrounds the digestive tract epithelium, digestive tubules, and gonads. These zoites were also observed in the hemolymph sinusoids of the gills, mantle, and adductor muscle. The zoites were oval or elongated and elliptical in shape, measuring 5–8 μm in length and 3–5 μm in width. They contained a round, eccentric nucleus (Figures 3(a) and 3(b)).

3.4. Presence of Bacteria. Bacilli (rod-shaped) and cocci (spherical or round-shaped) bacteria were observed in various parts of some mussels, including the gills, mantle, connective tissues near gonads, digestive tubules, and digestive epithelium (Figure 4(a)–4(d)). Some bacteria may have been decay bacteria since some samples (four samples) were moribund (collected from “unhealthy” lines that were deemed affected by summer mortality).

3.5. Parasitic and Pathogenic Identification. Prevalences of *P. olseni* and APX-infected animal samples were 47% ($n = 7$) and 67% ($n = 10$), respectively (Figure 5). Among the mussels analyzed, 60% ($n = 9$) of the sample were found to be associated with both bacilli and cocci bacteria (Figure 5). Out of seven unhealthy mussels, six samples (86%) were infected with *P. olseni*, whereas only 13% of healthy samples were infected with *P. olseni*. On the other hand, the same number ($n = 5$, prevalence = 71%) of unhealthy mussels were infected with

TABLE 1: Assessment of bacterial presence in unhealthy and healthy mussels using H&E, Gram, Ziehl Neelsen, and Giemsa stains.

Mussel groups	Sample ID	Bacteria	Stains			
			H&E	Gram	Ziehl Neelsen	Giemsa
Unhealthy	0418-I1	Rods	Y	Y	Y	Y
		Cocci	Y	Y	Y	Y
	0418-I2	Rods	N	Y	Y	Y
		Cocci	N	N	N	N
	0418-I3	Rods	Y	Y	Y	Y
		Cocci	Y	Y	Y	Y
	0418-I4	Rods	N	N	N	N
		Cocci	N	N	N	N
	0418-I8	Rods	Y	Y	N	Y
		Cocci	N	N	N	Y
	0418-I9	Unspecified	Y	N	N	N
		Rods	Y	N	N	Y
	0418-I10	Cocci	N	N	N	N
		Rods	N	N	N	N
Healthy	0418 H1	Rods	N	N	N	N
		Cocci	N	N	N	N
	0418 H3	Rods	N	N	Y	N
		Cocci	N	N	N	N
	0418 H4	Rods	Y	Y	Y	Y
		Cocci	N	N	N	No
	0418 H5	Rods	Y	Y	Y	Y
		Cocci	N	N	N	N
	0418 H6	Rods	N	N	N	N
		Cocci	N	N	N	N
	0418 H7	Rods	N	N	N	N
		Cocci	N	N	N	N
	0418 H8	Rods	N	N	N	N
		Cocci	N	N	N	N
0418 H10	Rods	N	N	N	N	
	Cocci	N	N	N	N	

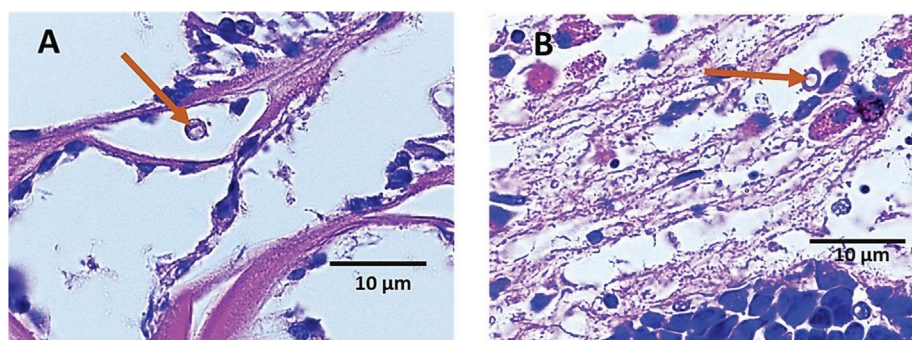


FIGURE 2: Histology slide staining with H&E showed trophozoites (orange arrows) of *P. olseni* in different tissues of *P. canaliculus*. (a) Gills (hemolymph sinusoids). (b) Connective tissue surrounding male gonads. Scale bars = 10 μ m.

APX and bacteria. Furthermore, APX and bacteria-infected healthy samples were 63% ($n=5$) and 38% ($n=3$), respectively. A Pearson's *chi*-square test showed a strong association between the health condition of mussels and the presence of *P. olseni* (p -value = 0.009). However, there were no significant associations found between the health condition of

mussels and the presence of APX (p -value = 0.573) or bacteria (p -value = 0.378).

3.6. Immunological Tissue Responses (Ceroid/Brown Material and Hemocytosis). In various tissues of *P. canaliculus*, such as mantle, gills, and connective tissue around gonads and

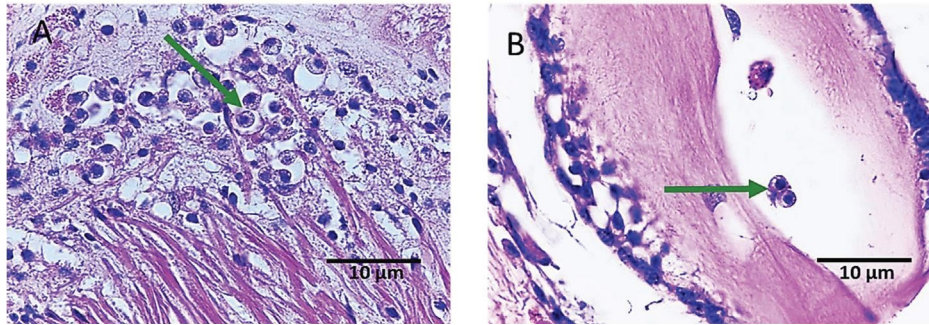


FIGURE 3: Histology slide staining with H&E showed APX (green arrows) in different tissues of *P. canaliculus*. (a) Connective tissue surrounding digestive epithelium. (b) Gills (hemolymph sinusoids). Scale bars = 10 μm.

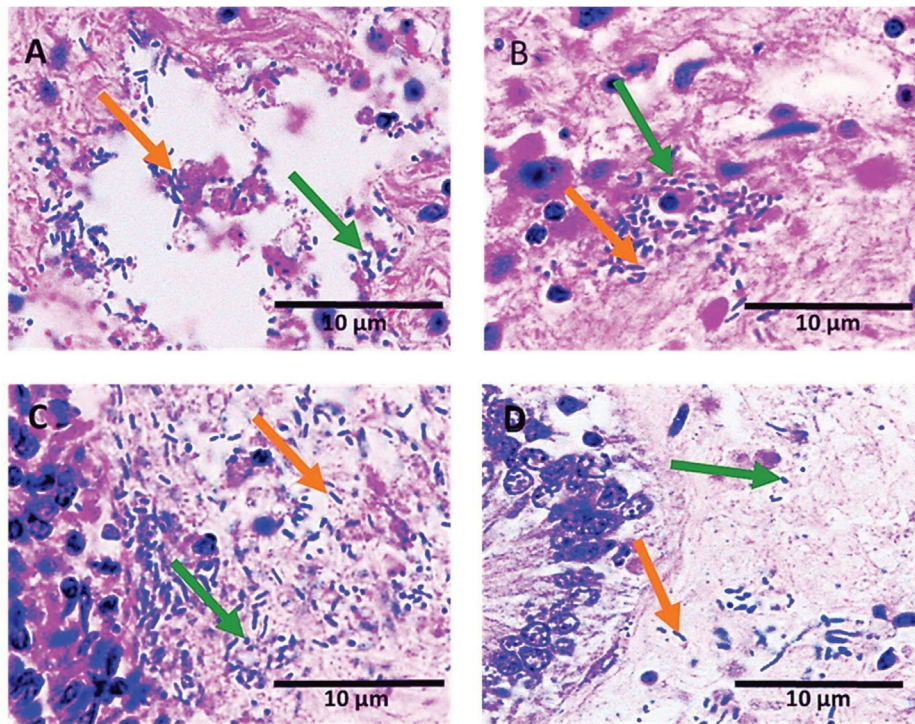


FIGURE 4: Histology slide staining with H&E showed bacteria (orange arrows and green arrows) in tissues of *P. canaliculus*. (a) and (b) Muscle tissues, (c) mantle, (d) connective tissues near digestive epithelium. Orange arrows show bacilli, while green arrows point to cocci-shaped bacteria. Scale bars = 10 μm.

digestive tubules, there was an accumulation of ceroid/brown material. This accumulation was recorded in light, moderate, and heavy amounts, as shown in Figures 6(a) and 6(b). Furthermore, hemocytosis was observed in various tissues of *P. canaliculus* (Figures 6(c) and 6(d)). Eighty percent of observed samples (12/15) showed tissues with a medium concentration of ceroid accumulation, while the high and low categories were observed at prevalences of 7% (1/15) and 13% (2/15), respectively. Haemocytosis was recorded in only 22% (5/15) of samples.

3.7. Assessment of Bacterial Presence Using Different Stains. Using ZN stain (53%, $n = 8$), mussels were seen to contain bacteria (Table 1). Bacteria were detected with Gram stain (44%, $n = 7$), Giemsa stain (44%, $n = 7$), and H&E stain

(40%, $n = 6$) (Table 1). Thus, H&E might be the least effective in staining bacteria; however, it might be the best staining method for general pathology and anatomy. By inspection, Giemsa provided the clearest visual definition of bacteria, although it was on a par with ZN. The differentiative ability of Gram and ZN-stains could not be assessed as no Gram-positive or acid-fast bacteria were seen in the slide preparations.

4. Discussion

A histological study was conducted in 2018 during a summer mortality event to detect pathogens and parasites and evaluate immunological tissue responses in healthy and unhealthy mussels. The study revealed that unhealthy mussels (those affected by summer mortality) had a higher prevalence of

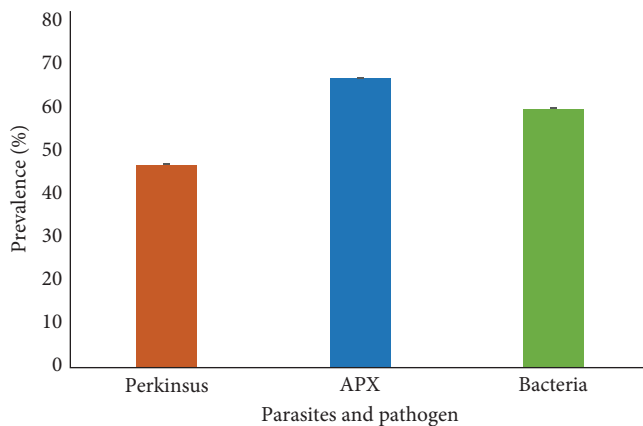


FIGURE 5: Prevalence (\pm SE) of parasites and pathogens associated with mussels ($N=15$) (prevalence is the number of hosts infected with one or more individuals of a particular parasite species divided by the number of hosts examined for that parasite species. It is commonly expressed as a percentage or proportion).

P. olseni, APX, and bacterial (rods and cocci) infection compared to healthy mussels. In our previous study, a metabolomics approach was used to compare the metabolite profiles of healthy and unhealthy mussels during the same summer mortality event [1]. The results revealed 41 metabolites significantly different between the two mussel groups, reflecting perturbations in energy metabolism, amino acid metabolism, protein degradation/tissue damage, oxidative stress, and antimicrobial responses. In addition, the flow cytometric analyses of mussel hemolymph showed a higher percentage of mortality, oxidative stress, and apoptosis in the hemocytes of unhealthy mussels compared to healthy mussels. The metabolic and immune responses suggested that these mussels may have suffered from pathogen infections during this mortality event, along with the stress caused by elevated temperature. Thus, the detailed histological assessment employed in the present study complements and corroborates that mussels were indeed affected by pathogens and parasites, including *P. olseni*, APX, and bacteria (rods and cocci), which could have resulted in the observed mortalities.

In this study, we have identified the parasite *P. olseni* by histology in *P. canaliculus*, which was collected from a mussel farm in Kaiaua (North Island, New Zealand). The prevalence of the parasite in the mussels was found to be 44%. In our previous study, *P. olseni* was also identified in *P. canaliculus* (prevalence 56%) collected from the same mussel farm, which was confirmed by *In-situ*-hybridization [10]. Therefore, the *Perkinsus*-like trophozoites seen in the study are indeed *P. olseni* based on our previous study. Furthermore, *P. olseni* was confirmed by histology and Ray's fluid thioglycollate medium from *P. canaliculus* in the South Island, New Zealand [11]. Previous research by Park et al. [46, 47] recorded 97%–100% of *P. olseni* infection in clams (*R. philippinarum*) in Ariake Bay, Kyushu, Japan, and Komsoe Bay (21°C in water temperature. In the present study, 86% unhealthy and 12.5% healthy mussels were infected with *P. olseni*. We also found that there was a highly significant

association between the health condition of mussels and the presence of *P. olseni*. Villalba et al. [48] noted a high prevalence of *P. olseni* infection in the clam *Tapes decussatus* during spring and summer when surface water temperatures expanded. Park et al. [46] found an incredibly elevated amount of *P. olseni* in clams in late summer in Gomsoe Bay, Korea. According to Yang et al. [49], food availability is somewhat low during late fall/autumn to winter, and during summer and early fall/autumn, spawning activities exhaust clams, which might hinder their defense capacity. According to Carella et al. [50], during the warmer seasons, the relatively higher temperature can encourage the growth and spread of *P. olseni*. Thus, *P. olseni* infections appear to be highly related to water temperature and the health condition of mussels, with high infection rates of *P. olseni* resulting in negative impacts on the population structure of *P. canaliculus*.

In this study, 71.4% of unhealthy mussels and 62.5% healthy samples were infected with APX, but there was no significant relationship between health condition and the presence of APX. APX zoites at high prevalence (63%) were recorded in various tissues of *P. canaliculus* gathered from North Island (Whakatiwai), New Zealand in 2018 [51]. Suong et al. [51] likewise noticed a high prevalence (60%) of APX in cultivated *P. canaliculus* from North Island (Coromandel), New Zealand, in 2016. Different researchers have recognized APX in *P. canaliculus* [5, 6, 52], Mediterranean mussels, *Mytilus galloprovincialis* [52], and hairy mussels, *Modiolus areolatus* [51]. According to Suong et al. [51], APX might be connected with the morbidity and mortality of mussels under specific pressure conditions. Subsequently, 62.5% infection of APX in mussels in the current study suggests that these healthy mussels were also stressed on this occasion. Suong [53] noted that biotic elements (e.g., host density, common microbes, and host formative stages) and abiotic components (e.g., temperature, pH, and salinity) can make APX to proliferate and cause harmful outbreaks in the New Zealand aquaculture industry. In this manner, ecological variables, for example, raised temperature, seem to improve the spread of APX infection and, in extreme cases, APX might be related with disease outbreaks.

The presence of bacteria (rods and cocci) was found in 53% samples (71.4% of unhealthy mussels and 37.5% of healthy samples). Where large numbers of bacteria were noted within tissues, it was difficult to ascribe their presence to pathology or to postmortem decay. Like APX, there was no significant relationship between health conditions and the presence of microorganisms. In the present study, most of the bacteria were found in moribund mussels. As per Webb and Duncan [6], stressed, dying, and dead creatures in some cases appear as microscopic organisms (mostly rods and cocci) which are connected with to changes in the autolysis and necrosis of the gill epithelial surface, mantle cavity, and organ surface of bivalves (*P. canaliculus*, *C. gigas*, *O. chilensis*, and *M. galloprovincialis*). Travers et al. [54] noted that bivalves are unfavorably impacted by numerous microbes and are related to mortality events of hatcheries and natural beds. According to Nguyen and Alfaro [1], within the summer season, aquaculture species may encounter heat pressure and

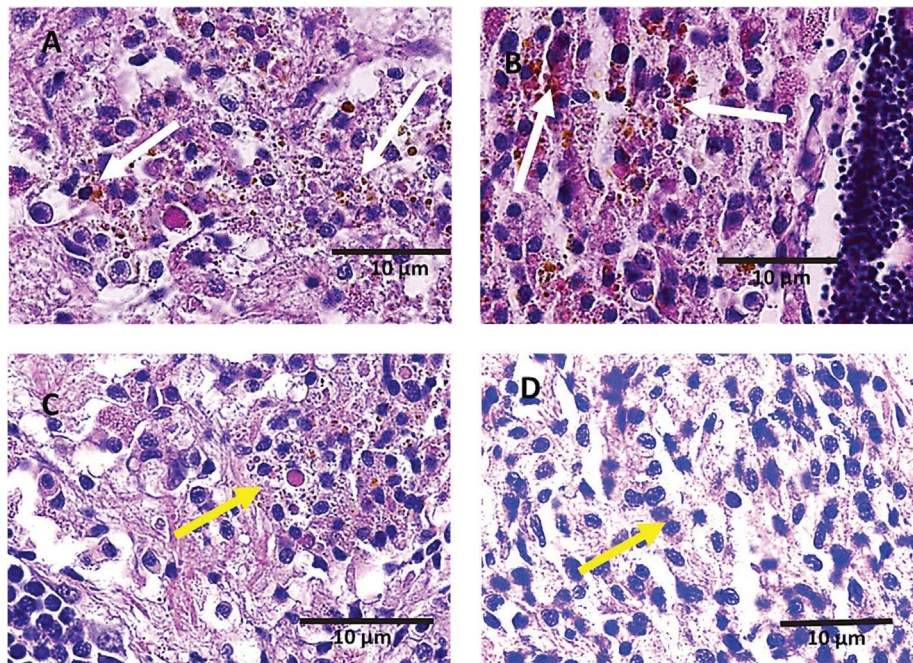


FIGURE 6: Ceroid/brown material accumulation and hemocytosis in tissues of *P. canaliculus*. Ceroid/brown material accumulation (white arrows) in mantle (a) connective tissue surrounding male gonads (b). Hemocytosis (yellow arrows) in connective tissue near male gonads (c) and hemocytosis in mantle (d). Scale bars = 10 µm.

microorganism loads, which tend to devastate their immune systems and cause mortality. Therefore, the higher percentages of bacteria in unhealthy mussels contrasted with healthy mussels in this study, is suggestive of a high stress level in the unhealthy mussels throughout the summer mortality event.

Haemocytosis, as an immunological response, was also observed in association with mussel tissues within the present study. Haemocytosis was also reported by Webb and Duncan [6] in New Zealand bivalve mollusks (*P. canaliculus*, *M. galloprovincialis*, *Crassostrea gigas*, and *Ostrea chilensis*). According to Webb and Duncan [6], hemocytosis can be an indication of infection of copepods or *Perkinsus* in *Perna canaliculus*. Muznebin et al. [10] recognized *P. olseni* trophozoites within the hemocytosis relationship with mussel (*P. canaliculus*) tissues and observed a significant positive affiliation between hemocytosis and *Perkinsus* infection in *P. canaliculus*. Couñago [55] reported that hemocytosis may occur with an inflammation process due to the presence of foreign particles. Therefore, hemocytosis seen in different mussels' tissues may indicate an unhealthy state of the animal. Oubella et al. [56] observed an increased number of hemocytes in the hemolymph of clam (*R. philippinarum*) found on the Atlantic coasts of Western Europe challenged with *Vibrio tapetis*, a pathogenic stimulus. According to Carballal et al. [57], hemocyte number was emphatically corresponded with water temperature in *M. galloprovincialis*, with the least number of hemocytes observed in winter and the most noteworthy in summer. These observations suggest that the incidence of hemocytosis within the tissues of mussels might be due to the combination of the presence of pathogens and parasites and the increase in water temperature.

The accumulation of ceroid (brown material) was noted in different organs (mantle, digestive glands, gills, and gonad) of *P. canaliculus* in this study. Couñago [55] also observed brown materials in the gills of clams, which were attributed as an indication of chemical aggravation from toxins and microorganisms. Muznebin [10] observed a noteworthy positive relationship between the brown material accumulation and parasites (*P. olseni* and APX). Neves et al. [58] likewise recorded ceroids within the digestive gland of snails (gastropod) presented to dinoflagellates. According to Apeti et al. [59], ceroid is common in oysters, and it might demonstrate cell stress. Carella et al. [60] stated that ceroid and lipofuscin aggregation have been connected with age and pollutant contact in mollusks. Thus, ceroid may be considered a response to immunological challenges or other stresses, such as temperature stress, transport pressure, and/or obscure components throughout the summer mortality event.

In this study, different histological stains, such as H&E, Gram, ZN, and Giemsa, were used to investigate the best stain for detecting and identifying bacteria in *P. canaliculus*. The findings showed that H&E was the best staining method for general pathological and anatomical characterization, while Giemsa provided the clearest visual definition of bacteria; Gram and ZN-stained specimens revealed no Gram-positive or acid-fast bacteria. According to Woods and Walker [37], existing forms of inflammation explained through H&E can prompt different knowledge on the infection status or wound, yet individual microbes are not effectively distinguished with H&E staining alone. Moreover, Gram stain is the most common staining technique to separate between Gram-positive and Gram-negative bacteria in various kinds

of tissues [37, 38]. Ulrichs et al. [40] noted that the determination of mycobacterial infection relies upon the ZN stain, which identifies mycobacteria due to the characteristic acid-fast cell wall arrangement and construction of the ZN stain. Furthermore, the bacilli (rod-shaped, Gram-positive) often appear in dim due to the ZN stained in tissue sections [61]. Therefore, the study had proven that shellfish histopathological assessment, as shown before, is best using H&E and Gram stains when looking for pathogens and identifying bacteria.

In conclusion, this study reports the presence of *P. olsenii* infection in healthy and unhealthy *P. canaliculus* throughout a summer mortality occasion. A histopathological examination of farmed mussels identified potential pathogens and parasites (*P. olsenii*, APX, and bacteria), as well as immunological tissue reactions (hemocytosis and ceroid material) in *P. canaliculus*, which could be used to evaluate host health status. A much higher prevalence of *P. olsenii* was recorded from unhealthy mussels than healthy mussels and there was a highly significant association between health condition and the presence of *P. olsenii* in mussels. Furthermore, higher prevalences of APX and bacterial (rods and cocci) infections were observed in unhealthy mussels than in healthy ones, but there was no correlation between health conditions and the presence of APX and bacteria. This finding suggests that *P. olsenii* could be a harmful agent for *P. canaliculus*, along with elevated temperatures during summer mortality events in New Zealand. Hence, there is a need for future studies to investigate the pathogeny of *P. olsenii* in mussels, as well as the complex interaction between *P. olsenii*, mussels, and temperature. We have also provided an assessment of different histology stains (H&E, Gram, ZN, and Giemsa). H&E appeared to be the best for general pathological and anatomical characterization, while Giemsa provided the clearest visual definition of bacteria. In this aspect, it was comparable to ZN in apparent sensitivity. Although Gram and ZN staining revealed bacterial cells marginally better than H&E, their differential staining could not be assessed as no Gram-positive or acid-fast bacteria were seen, and no mussel-positive controls were available for comparison. Although this study also provides an illustrated guide to some significant mussel health indicators, for future studies, other diagnostic techniques will be used, such as immunohistochemistry, electron microscopy, and PCR, to strengthen this type of research.

Data Availability

All data generated or analyzed during this study are included in the article.

Conflicts of Interest

The authors have no relevant monetary or nonmonetary interests to disclose.

Authors' Contributions

Farhana Muznebin has done the conceptualization, examination, methodology, sampling, formal analysis, data curation, roles/writing—original draft. Thao V. Nguyen has done

the sampling and writing—review and editing. Andrea C. Alfaro has done the conceptualization, writing—review and editing, resources, funding acquisition. Stephen C. Webb has done the conceptualization, examination, methodology, writing—review and editing.

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