Bacterial Infection in Tasmanian Farmed Abalone: Causes, Pathology, Farm Factors and Control Options

JUDITH HANDLINGER, JEREMY CARSON, LINDA DONACHIE, LES GABOR AND DAVID TAYLOR
Fish Health Unit of the Tasmanian Aquaculture & Fisheries Institute and DPIWE Animal Health Laboratory, Tasmania, PO Box 46, Kings Meadows, Tasmania, 7249, Australia

ABSTRACT

Abalone aquaculture based on artificial feeds and intensive generally land-based systems is increasing in Australia. Intensive systems are vulnerable to diseases of poor environment control. This paper reports several types of bacterial diseases investigated in grow-out stock of *Haliotis rubra, H. laevigata* and their hybrids over approximately 10 years, their pathology, farm factors precipitating disease outbreaks, and the potential for disease control using antibiotics. *Vibrio* species infections have been the most common cause of infection, with a variety of species isolated from moribund animals, generally irrespective of the primary insult. Specific disease outbreaks, with differing pathology, have been associated with two species, *Vibrio harveyi* and *V. splendidus* I. Specific pathology has also been associated with *Flavobacterium*-like species. In most cases, stress factors precipitating disease has been identified, and control has been largely directed to eliminating this stress. Antibiotics have also been used, with equivocal results, precipitating preliminary studies of antibiotic absorption and efficacy in these species. The limited potential for antibiotic use, and the requirement for understanding and control of on-farm stress, is discussed.

INTRODUCTION

Despite major wild abalone industries, there are few described diseases of abalone. With the emergence of abalone aquaculture industries, more diseases are to be expected, partly due to closer observation, and partly as the more crowded conditions of animals under culture are more likely to favor disease spread. That the majority of described molluscan diseases are major primary pathogens is partly due to inappropriate animal translocation, but this also reflects the relatively natural food and environment which extensive sea-based bivalve culture systems provide. In contrast, land-based abalone cultured on artificial feeds, and tank held wild caught stock, are also vulnerable to diseases of poor environment. These are often expressed as infections with organisms which are probably ubiquitous.

Understanding the pathogenesis of these diseases and farm factors precipitating disease outbreaks will assist in control through early recognition and correction of the underlying husbandry faults. This paper reports characteristics of bacterial disease investigated over

Handlinger, J., J. Carson, L. Donachie, L. Gabor and D. Taylor. 2005. Bacterial infection in Tasmanian farmed abalone: Causes, pathology, farm factors and control options. *In* P. Walker, R. Lester and M.G. Bondad-Reantaso (eds). Diseases in Asian Aquaculture V, pp. 289-299. Fish Health Section, Asian Fisheries Society, Manila.

10 years in Tasmanian grow-out stock of Blacklip (*Haliotis rubra*), Greenlip *(H. laevigata*) and their hybrids.

MATERIAL AND METHODS

Abalone ranging upward from 1 cm shell size were received either live or fixed whole in seawater formalin as part of routine diagnostic investigations of mortality occurring in aquacultured and occasionally wild harvest stock in temporary holding. Live animals were examined using a dissecting microscope and surface lesions were examined by phase contrast microcopy of wet smears from areas of pallor. For histopathology, tissues or whole juvenile abalone were fixed in seawater formalin, after benzocaine euthanasia and shell removal. After 24 hours fixation, haematoxylin and eosin stained sections including visible lesions, foot, mantle, mouth, gill, heart, kidneys, stomach, and digestive system, were prepared by standard methods.

For microbiology, affected tissue or haemolymph were inoculated onto TCBS agar for *Vibrio* species, Johnson's marine agar (a variant of ZoBell's 2216), and selective and non-selective marine Shiehs medium for the *Flexibacter-Flavobacterium-Cytophaga*-like group of bacteria (FFCLB). Smears for Gram stain were also prepared.

Culture plates were incubated at 25°C for three days. *Vibrio* isolates were identified using MicroSys(TM) *Vibrio* test panels and probabilistic identification software. The test panel comprises 48 conventional phenotypic tests and the reference database is regularly updated to include data for 56 species in the genera *Vibrio, Photobacterium* and *Moritella*. Yellow pigmented spreading and non-spreading colonies from marine Shieh's medium were characterised by cell morphology, Gram reaction, oxidase and acid production from glucose. Colonies suggestive of *Tenacibaculum maritimum* were identified by PCR using a 16S rRNA primer set (Carson, 1998; Wilson, *et al*., 2002).

Table 1. Characteristics of *Vibrio splendidus* I and *Vibrio harveyi* isolated from diseased abalone.

	Vibrio splendidus I		*Vibrio harveyi*	
Test	01/2567	Expected	03/0411	Expected
Swarming	-	-	-	13
Growth in 0% NaCl	-	-	-	-
Thornley's Arginine	+	+	-	-
Moller's Lysine	-	-	+	+
Moller's Ornithine	-	-	-	96
Nitrate reduction		+	+	++
Oxidase	+	+	+	+
Indole	+	+	+	+
ONPG	+	751	-	67
Voges Proskauer		-	-	- -
Resistance to O129 10 µg .	-	-	+	71
Resistance to O129 150 µg	-	-	-	17
Resistance to Ampicillin 10 µg	-	-	-	92
Resistance to Polymyxin B 50 iu	-	-	-	13
Resistance to Tellurite (0.0005%)	-	-	-	-
Aesculin hydrolysis	+	50	+	53
Alginase	-	50	+	38

Urease		-	-	-	50
Fermentation:	L-arabinose	-	-	-	-
	Arbutin acid	-	17	-	71
	Mannose acid	+	75	+	+
	Salicin acid	-	-	-	88
	Sorbitol acid	-	17	-	4
	Sucrose acid	-	67	-	29
	Mannitol acid	+	+	+	+
Utilisation:	L-arabinose	-	-	-	-
	Cellobiose	+	+	+	+
	D-galactose	+	75	-	58
	D-glucose	+	+	+	+
	D-mannose	+	75	+	+
	Melibiose	-	-	-	8
	Lactose	-	-	-	-
	Melizitose	-	50	-	-
	Sucrose	-	50	-	25
	Trehalose	+	+	+	+
	Xylose	-	-	-	-
	Ethanol	-	-	-	4
	Glycerol	+	+	-	71
	Propanol	-	-	-	13
	D-sorbitol	-	50	-	4
	Gluconate	+	25	+	97
	D-glucuronate	-	75	+	89
	Amygdalin	-	-	-	8
	Arbutin	-	-	-	33
	L-citrulline	-	75	-	8
	L-hydroxy proline	-	-	-	17
	L-leucine	-	-	-	-
	D-glucosamine	-	-	+	4
	DL-3-hydroxybutyrate	-	-	-	-
	(-ketoglutarate	-	+	-	75
	Succinate	+	+	+	88
Identification		*V. splendidus I*		*V. harveyi*	
Willcox probability score		0.997		0.999	

[1]Percent strains positive

RESULTS

There was no obvious species difference in susceptibility to the bacterial diseases observed, and the results combine findings from both species and their hybrids.

Septicaemic vibriosis

Vibrio species were commonly associated with moribund abalone, irrespective of the primary insult. Spread within contact populations has so far been associated with outbreaks of only two Vibrio species, *V. harveyi* and *V. splendidus I* (see Table 1 for typical characteristics), the pathology of which is described below. Outbreaks typically occurred as peaks of mortality in summer, with few gross or clinical signs of infection, following a sharp increase in water temperature, or other stresses related to handling or water quality.

Frequently, low levels of mixed Vibrio species were isolated. Other Vibrios isolated and of pathogenic potential included V. tubiashii, V. anguillarum, and recently V. tapetis from a mixed infection in a wild caught abalone. Many Vibrio isolates could not be speciated.

V. splendidus I

One sustained outbreak of *V. splendidus I* was studied, though this was also a common sporadic isolate from moribund animals. On two other occasions this was consistently isolated from all animals in an affected group, sometimes as part of a mixed infection. However these outbreaks were not sustained.

The studied outbreak showed similar pathology to most septicaemias, and had the following characteristics: As well as being detectable in haemolymph, bacteria were typically detectable, often in large numbers, in the peri-gut haemocyte region. Selective degeneration of the peri-gut haemocytes despite an intact gut epithelium, distinguished the infection from peri-mortem invasion, but not necessarily from other *Vibrio* infections (Figure 1). Localization occurred less frequently within other vascular beds such as heart muscle which appeared selectively targeted by this species (Figure 2), gills, left kidney, or connective tissue round major ganglia (Figure 3). Localization was often intense in the left kidney, which appeared as a site of selective filtration. Bacteria were often trapped within protein deposits, which were common in the gills as well as left kidney, and were occasionally seen in other locations. Light focal haemocyte responses were seen in tissues such as the heart muscle, but were generally minimal. Affected peri-gut interstitial tissue frequently appeared oedematous, occasionally hypercellular, suggesting acute and chronic stages of the infection. Very large numbers of bacteria were occasionally present here, despite low numbers in haemolymph, suggesting this is a site of selective entrapment.

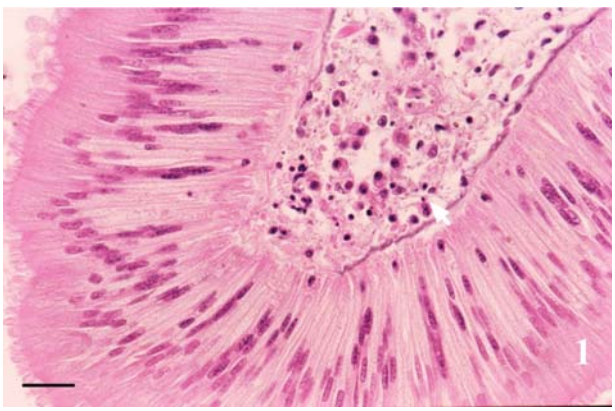


Figure 1. Degeneration of haemocytes (arrow) under intact gut epithelium associated with septicaemia, in this case with *Vibrio harveyi* infection. (Scale bar = 20 mm)

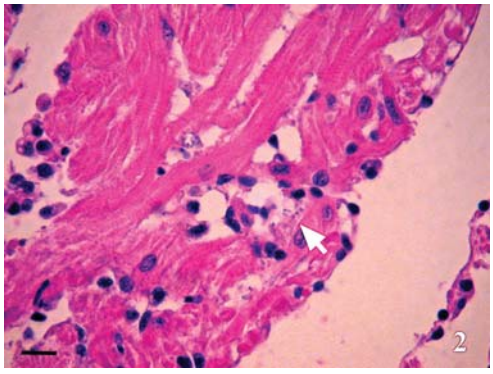


Figure 2. Inflammation and bacteria (arrow) within the ventricular myocardium of juvenile Greenlip abalone with *V. splendidus I* infection. (Scale bar = 10 mm)

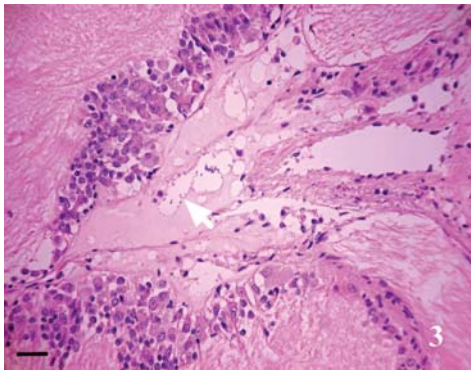


Figure 3. Bacteria within protein rich fluid surrounding a major ganglia. Same abalone as Fig 2. (Scale bar = 20 mm)

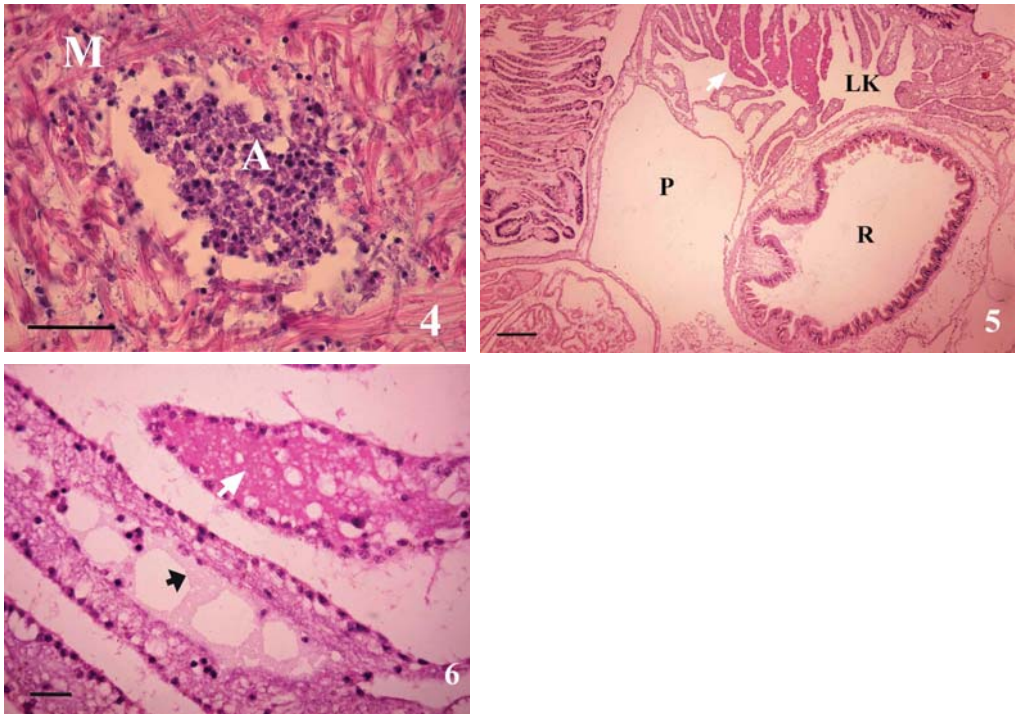
No specific predisposing factors, and no specific relationship to temperature, have so far been identified for initiating *V. splendidus* infection. However the prolonged outbreak occurred during winter, while infections at other times were not sustained.

V. harveyi pustule disease

Outbreaks typically occurred after exposure to summer temperatures, and prolonged outbreaks were often precipitated by short periods of stress, such as several hours of interruption to water supply and resulting transient increase in temperature.

Infection with V. harveyi was similar to V. splendidus infections in the septicaemic phase, but differed from other Vibrio infections by a more marked tissue response generally leading to the formation of pustules that are analagous to abscesses in vertebrates. In this study we refer to large aggregations of live and necrotic haemocytes into an organised lesion as an abscess. Abscesses were frequently observed in the foot muscle, with many bacteria surrounded by a well developed haemocyte response (Figure 4). There was little evidence of encapsulation by other than the surrounding haemocytes. This infection was characterized by prolonged outbreaks or a recurrence with recurrent stress, apparently due to re-activation from these abscesses.

Two distinguishable strains of *V. harveyi*, both associated with abscess formation, differed in the degree of localization in the peri-visceral granulocyte layer, and the degree of association with pycnosis and fragmentation of intravascular and extravascular haemocytes (Figure 1). Protein precipitation and bacterial concentration in the left kidney were marked and consistent with both bacterial strains (Figures 5 and 6). Kidneys showing marked eosinophilia from protein precipitation and basophilia from bacterial aggregates in haematoxylin and eosin stained sections were an excellent histological marker for probable infection.



Figures 4-6. The same juvenile abalone from a sustained outbreak of V. harveyi infection. Fig 4 shows an abscess (A) within the foot muscle (M), scale bar = 100 mm. Fig 5 shows protein precipitation (white arrow) in several of the folia of the left kidney (scale bar = 200 mm). Fig 6 is a higher magnification of the same kidney, showing diffuse protein deposition as intense eosinophilia (white arrow) in one of the folia, and masses of bacteria in the central vessel of another (black arrow). (Scale bar = 20 mm)

Figures 7. "Blister" like lesions associated with vibriosis, especially *V. harveyi* infection. Fig 7 shows the gross lesions (arrow) in wild caught Blacklip abalone in temporary holding. (Scale bar = 1 cm).

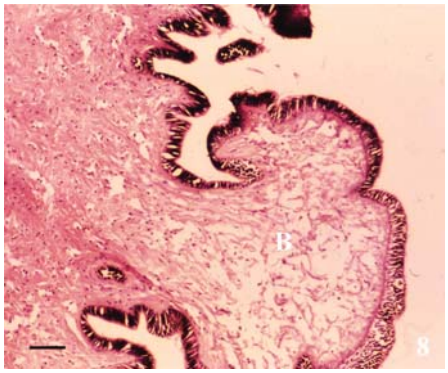


Figure 8. "Blister" like lesions associated with vibriosis, especially *V. harveyi* infection. Fig 7 shows the gross lesions (arrow) in wild caught Blacklip abalone in temporary holding. (Scale bar = 1 cm), Fig 8 the histological appearance (scale bar = 50 mm).

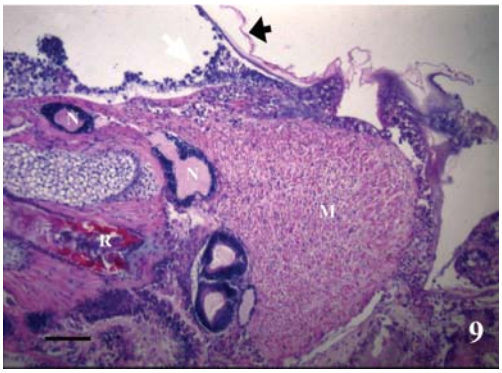
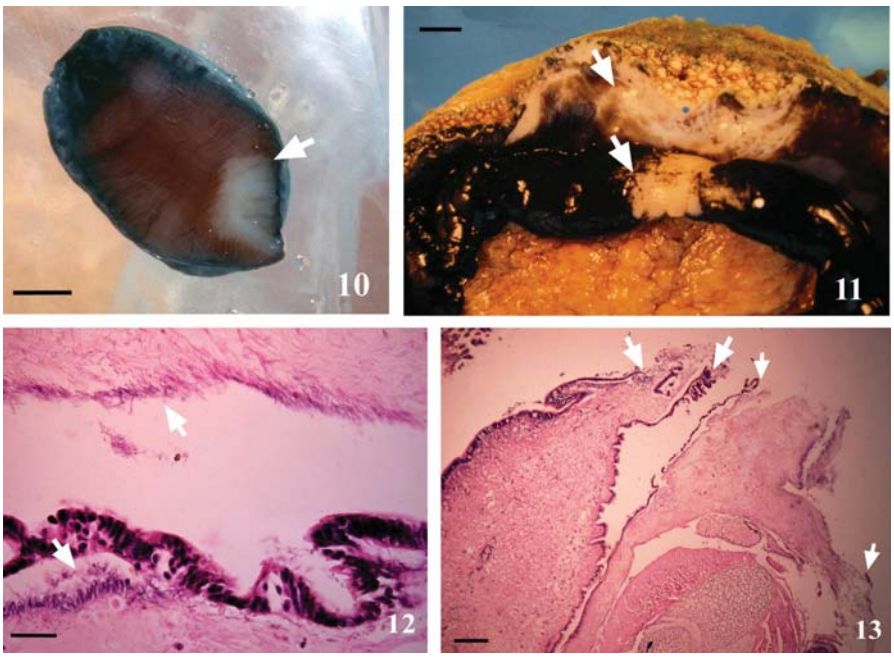
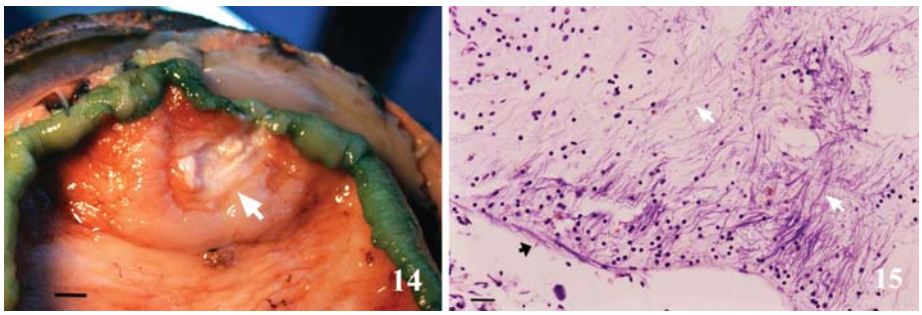


Figure 9. Surface damage leading to mixed bacterial infection (arrow) and inflammation of early juvenile following exposure to the fungicide OBPA (10.10'-oxybis-10H-phenoxarasine). S = shell, M muscle, N nerve, R radula. (Scale bar = 50 mm).



Figures 10-13. *Flavobacterium* syndrome 1.
Figure 10. Gross appearance of pigment loss (arrow) in juvenile Greenlip abalone, termed "white foot" by farms. **Figure 11.** of wild caught adult Blacklip, more clearly shows the shallow nature of the lesions, limited to loss of the pigmented epithelium (Scale bar = 1 cm). Under-running of epithelium by the bacteria, resulting in lifting and loss are shown in the two adjacent sections of **Figure 12.** (Scale bar = 20 mm). **Figure 13.** Juvenile abalone showing large areas of superficial bacterial invasion and epithelial loss between pairs of small and large arrows. (Bar = 20 mm).



Figures 14-15. *Flavobacterium* syndrome 2.
Figure 14. Deep surface necrosis, adult wild caught Greenlip from the summer outbreak.
Figure 15. Superficial (black arrows) and deep bacterial invasion (white arrows), by large sometimes beaded bacteria, with minimal visible host response. (Scale bar = 20 μm)

Abscess formation within foot muscle was the most characteristic lesion, though focal fluid pooling within the foot was also common. This presented grossly as surface "blisters" (Figure 7). These are recognized by farms as an indicator of infection, usually but not always with *V. harveyi*, leading to their term of "blister disease" for this infection. Bacteria were only occasionally present in blisters in numbers sufficient to be detectable by normal light microscopy, and cell responses were minimal, the appearance being that of localized oedema, with apparent rupture of fibres (Figure 8). Vacuolation of epithelium over these areas was common, often followed by loss of epithelium and rupture of the vesicle.

Secondary surface infection

Surface infection with mixed *Vibrio*-like bacteria was seen following shell damage by spionid polychaetes, and exposure to plastic incorporating the fungicide OBPA (10.10'-oxybis-10H-phenoxarasine), which is commonly used in plastics such as swimming pool liners (Figure 9). Two syndromes of surface infection with *Flavobacterium*-like bacteria, were also seen.

Flavobacterium syndrome 1

Presenting signs of loss of pigmentation and contact avoidance were seen on at least nine occasions, including six tank outbreaks (Figures 10 and 11). These lesions progressed to pale flaking epithelium and shallow epithelial erosions of the foot, epipodium, occasionally the mantle and superficial mouth parts, with masses of filamentous bacteria on the surface and under-running and replacing the epithelium (Figure 12). There was limited superficial penetration by bacteria and minimal host response. Coalescence of lesions often resulted in large areas being denuded of epithelium (Figure 13).

Smears from eroded surfaces showed a mat of debris containing slender filamentous *Flavobacterium*-like bacteria, often in large numbers, plus debris and other bacteria. A range of yellow pigmented colonies were isolated from sites of erosion in which filamentous bacteria were visible in smears or from haemolymph. On marine Shieh's medium, typical colony types were 1-3 mm in diameter, smooth, butyrous and non-adherent. The bacteria appeared as filamentous Gram negative rods, oxidase positive some of which fermented

glucose. There was a marked disparity in cell morphology in isolates between relatively short slender rods, 3-5mm long to robust cell types 1.0-1.5 mm wide and 8-10 mm in length. The variation in cell type was also reflected in colony pigmentation which varied between pale to deep golden yellow. Recovery rates were variable and ranged from light to heavy, sometimes represented by a single colony type or mixed colonies discernible by variable size and pigmentation. No further characterisation was undertaken but on the basis of culture conditions, colony and cell morphology, bacteria of this type are consistent with the *Flexibacter-Flavobacterium-Cytophaga*-like group of bacteria. Most isolates could not be speciated. In some instances colonies typical of *Tenacibaculum maritimum* (formerly *Flexibacter maritimus*) were detected and their identity was confirmed by 16S rRNA PCR. Such outbreaks occurred during the cooler months of May to October, when temperatures were 11 to 16°C, well within the range of these species. Initiating stress factors were generally traumatic, or in one case caused by high salinity, suggesting compromised healing and secondary infection at a time when host defenses may be reduced. Subsequent spread within tanks suggests bacterial load is also important.

Flavobacterium syndrome 2

More aggressive bacterial invasion was seen in one February outbreak in wild Greenlip abalone held at high stocking densities in tanks for 4-8 days at 19-21°C, conditions which are outside the species optimum (Edwards, 1996). Erosions progressed rapidly within one to several days to deep necrotic lesions (Figure 14). Larger and occasionally beaded *Flavobacterium*-like bacteria were present throughout affected sections, sometimes in association with other bacteria, and penetrating deeply into the expanding zone of necrosis (Figure 15). Host response was generally minimal. No consistent lesions were detected in other organs.

DISCUSSION

Vibriosis due to *Vibrio splendidus* has not previously been described from abalone of these age groups, but the non-specific pathology may make many cases indistinguishable from terminal opportunist infections. Nor has toxicity to molluscs been described for exposure to plastics containing the fungicide OBPA, though similar toxicity to salmon has been described once previously (Zitko *et al*., 1985). In both situations the presence of the fungicide made use of swimming pool liners inappropriate for aquaculture use, at least until leaching had reduced the fungicide levels.

Mortality associated with white focal foot lesions due to *V. harveyi* (reported as *V. 'carchariae'*, a junior synonym of *Vibrio harveyi*) has been described from Japan (Nishimori *et al*., 1998) and France (Nicolas *et al*., 2002). The pathology of *V. harveyi* infection in Tasmania resembles published reports of abalone pustule disease in China due to *V. fluvialis* II (Li *et al*., 1998), although there is a general lack of publication of detailed aspects of abalone pathology such the protein precipitates seen in gills, left kidney and other organs. Though the origin and significance of such precipitates is uncertain, they are apparently an indication of tissue damage, as this is also observed alone with non-infectious insults.

Although other *Vibrio* species were isolated, their occurrence was isolated and probably opportunistic, or may represent normal bacterial carriage as low levels of mixed *Vibrio* species were also isolated from clinically normal animals held for experimental trials (Handlinger *et al*., 2002).

Flavobacterium infections of abalone resemble those of fin-fish and have been briefly described previously (Handlinger *et al*., 2001b), and seen recently in New Zealand (B. Diggles, pers com). Although there were apparent differences in bacterial morphology, clinical expression, pathology and initiating stress factors between the summer and winter outbreaks, it is uncertain if these differences are due to bacterial type, or to a combination of elevated bacterial activity at high temperatures and reduced host responses with stress and with low temperatures. Nor do bacterial characteristics determine at this stage if a single type of FFCLB is causing the problem. On the evidence so far it is probably a syndrome that involves a range of similar opportunist bacteria of the FFCLB group. Further work is required to characterize fully this poorly described group of bacteria.

The potential devastating losses from these infections makes antibiotic use potentially valuable, especially where pustules result in on-going disease. However a preliminary trial of antibiotic absorption and efficacy in *H. rubra* suggests the value may be limited (Handlinger *et al*., 2001a; 2002). There was moderate absorption of oxytetracycline (OTC) from in-feed treatment, but poor absorption of sulphadiazine, amoxicillin and the quinolone oxolinic acid. Only oxolinic acid was well absorbed by bath treatment. Prolonged residue times with OTC and an uncertain future for quinolones in aquaculture reduce their usefulness. OTC was only partially effective against *V. harveyi* infection, and infection persisted within the population; trimethoprim was ineffective. Innate soluble antibacterial factors varied significantly between groups of abalone, suggesting variable susceptibility may be significant and may offer better avenues for control and monitoring. Thus antibiotic treatment appears at best short-term, with significant environmental and food safety regulation concerns.

CONCLUSIONS

It was concluded that abalone react differently to different bacterial pathogens, which has implications for disease control, especially for *V. harveyi* and similar bacteria which result in abscess formation leading to persistence of infection in the population. Disease control by the use of antibiotics is problematic, and future emphasis should be on other means of control, including management factors.

As environmental and management are significant factors in establishing infections, disease investigation is of more immediate importance to abalone farmers than to most bivalve mollusc culture, as is more detailed information of mollusc immune responses and of factors which may affect these.

REFERENCES

Carson, J. 1998. Development of molecular probes for use in bacterial disease diagnosis and health monitoring of farmed and wild finfish in Australia. Final Report on Project 93/128, Fisheries Research and Development Corporation, Canberra.

Edwards, S. 1996. Thermal preferences and tolerances of greenlip and blacklip abalone. Proceedings of the 3rd Annual Abalone Aquaculture Workshop, Port Lincoln, SARDI, Adelaide, pp.65-67.

Handlinger, J., Harris, J., Carson, J. and Taylor, D. 2001. Investigation into the potential use of antibiotics for disease control in abalone: A preliminary report. *In* Proceedings of the 8th Annual FRDC Abalone Aquaculture Workshop, July 2001, Fremantle, Western Australia. pp. 83-96.

Handlinger, J., Taylor, D. and Carson, J. 2001a. *Flavobacterium*-like infection of abalone. (Abstract). In Proceedings of the European Association of Fish Pathologists Tenth International Conference, September 2001. P P-209.

Handlinger, J., Carson, J. Harris, J and Taylor D. 2002. Antibiotic treatment of *Vibrio harveyi* infection in *Haliotis rubra. In* Proceedings of the 9th Annual FRDC Abalone Aquaculture Workshop, July 2002, Queenscliff, Victoria. pp 1-12.

Li, T.W., Ding, M. Zhang, J. Xiang, J. and Liu, R. 1998. Studies on the pustule disease of abalone (*Haliotis discus hannai* Ino) on the Dalian coast. Journal of Shellfish Research 17, 707-711.

Nicolas, J.L., Basuyaux, O., Mazurié, J. and Th_bault, A. 2002. *Vibrio carchariae*, a pathogen of the abalone *Haliotis tuberculata*. Diseases of Aquatic Organisms 50, 35-45.

Nishimori, E., Hasegawa, O., Numata, T. and Wakabayashi, H. 1998. *Vibrio carchariae* causes mass mortalities in Japanese abalone, *Sulculus diversicolor supratexta*. Fish Pathology 33, 495-502.

Wilson, T., Carson, J. and Bowman, J. 2002. Optimisation of one-tube PCR-ELISA to detect femtogram amounts of genomic DNA. Journal of Microbiological Methods 51, 163-170.

Size 7.25 x 10 inches