ABALONE AQUACULTURE SUBPROGRAM: THE POTENTIAL FOR ANTIBIOTIC USE IN ABALONE FOR DISEASE CONTROL.

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NON TECHNICAL SUMMARY

2000/206 **Abalone Aquaculture Subprogram: The Potential For Antibiotic Use In Abalone For Disease Control**.

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OBJECTIVES:

1. To confirm the potential for antibiotic control of bacterial infections of abalone
2. To determine which antibiotics are most suitable for use in abalone aquaculture and appropriate mechanisms of delivery.
3. Coincidentally to increase understanding of factors affecting *V. harveyi* infections in abalone, and provide tools for further research into its control.

NON TECHNICAL SUMMARY:

Industry desire for antibiotic use in abalone arose with the emergence of bacterial infection in aquaculture facilities, especially *Vibrio harveyi* in juveniles following stresses such as high summer temperatures. Bacterial diseases such as *V. harveyi* have the potential to cause major losses for abalone farming, especially if sequestrated in abscesses that can become sources of further infections. Under these circumstances, antibiotic use was considered appropriate, provided that this did control the disease and remove or reduce the risk of future outbreaks; that this use is sustainable; and that there was information to provide a legal framework for such use as no antibiotics were registered for use in abalone and anticipated legal changes would reduce option for off-label use. Because of small potential markets, pharmaceutical companies would not have been prepared to finance appropriate studies to obtain registration in Australia, and no similar studies appeared to be undertaken elsewhere, as few developed countries culture molluscs intensively.

In order to determine if any antibiotics warranted the significant cost of providing an on-going legal framework through registration or Minor Use Permits, factors affecting antibiotic use were reviewed and a series of preliminary investigations undertaken to confirm antibiotic absorption and efficacy. The aim was to determine whether antibiotic use as a component of bacterial disease control in abalone is feasible. In particular, there was concern that there was little information on antibiotic efficacy in seawater-based environments, that their use may be impractical due to difficulties of administration, that such use has significant implications for residue control, and that frequent use may result in antibiotic resistance.

The questions of adequate absorption and primary residue clearance rate were addressed first, except for trimethoprim for which a suitable analytical test for tissue levels was not available.

Potential for antibiotic use in abalone

The most promising antibiotic from these trials (oxytetracycline), plus trimethoprim, were then tested for efficacy against the current most problematic pathogen, *Vibrio harveyi.*

Objective 1: To confirm the potential for antibiotic control of bacterial infections of abalone.

In an efficacy trial against a natural *V. harveyi* outbreak in *Haliotis rubra* (Leach), oxytetracycline reduced overall mortality, though deaths ceased in both treated and untreated groups during the experiment and remaining treated and untreated animals continued to carry bacteria. Trimethoprim was ineffective when used alone. Initial trials of oxytetracycline against artificial infections using graded injections of *V. harveyi* had been less successful, demonstrating the limitations of artificial infections for such trials, and of antibiotic use against overwhelming infections.

Objective 2: To determine which antibiotics are most suitable for use in abalone aquaculture and appropriate mechanisms of delivery.

Absorption was assessed for antibiotics selected from groups registered for use in other aquatic animals, using three administration methods (bath treatment, milled into artificial feed, and coated onto feed in agar). As the primary objective was to verify whether adequate absorption could be obtained, rather than to define an optimum dose rate, the antibiotics were generally administered at a dose equivalent to or greater than the maximum reported dose rate used to treat other aquatic animals. A higher level was used where absorption failure was strongly suspected.

Tissue levels measured at the end of the treatment period and one week later are reported for oxytetracycline, amoxicillin, oxolinic acid, and a trimethoprim / sulphadiazine mixture (potentiated sulphonamide) measured as the sulphonamide level. Absorption was generally poor except for moderate oxytetracycline levels from in-feed treatment and high levels of oxolinic acid from bath treatment. Moderate levels of at least the sulphonamide component were also found from the potentiated sulphonamide bath treatment, warranting investigation of whether the potentiator in this mixture (trimethoprim) was well absorbed by inference from the *in vivo* efficacy trial. In light of the negative results obtained with the use of this antibiotic against *V. harveyi*, when used alone at single-treatment levels, it appears unlikely that this potentiated sulphonamide mixture would be useful against this or similar infections. Oxolinic bath treatment showed promise for stock classes in which such treatment is practical, but is no longer allowable in production animals due to human health concerns. Testing of more acceptable quinolone derivatives could be warranted. Thus oxytetracycline administered in feed remains the only antibiotic with demonstrated absorption and efficacy for treatment of this type of infection.

One-week residues were significant only for oxytetracycline, which is consistent with the slow residue clearance of this antibiotic in other animals, and previous field experience in abalone Residue clearance is therefore an issue which will limit suitability of such treatment for animals close to marketing, and may generate costs in residue testing.

This set of experiments also compared delivery systems. For all antibiotics tested, tissue levels at one day post treatment were reduced when the antibiotic was coated onto the food, compared with the same dose rate incorporated into the food. This effect was detectable even when measurable tissue levels were very low (amoxicillin, sulphonamide from the potentiated sulphonamide mixture, and oxolinic acid).

Part of this reduction appeared due to reduced palatability, as intake was reduced by all antibiotic treatments, and by the agar coating alone, compared to consumption of uncoated, untreated food subjected to the same preparation processes. The reduction in consumption was significant for both treatments and method of application. For oxytetracycline and the potentiated sulphonamide mixture, which were relatively well tolerated in food, this palatability reduction was increased when the antibiotic was administered by agar coating rather than in-feed. For amoxicillin and oxolinic acid, which appeared highly unpalatable in feed, and for trimethoprim which was moderately well tolerated in feed, intake of agar coated food treatment was slightly higher than with in-feed treatment. Despite these food intake levels, all antibiotics showed lower tissue levels from coated treatments, which indicates that as well as reduced palatability, external factors such as leaching and / or inactivation are likely to be involved, to levels varying with antibiotic chemistry. For the most useful antibiotic, oxytetracycline, administration by agar coating of pellets resulted in a reduction in food intake to only 37% of the in-feed treatment (approximately 25% of the intake from uncoated untreated food). One-day post-treatment tissue levels were only 16% of those from in-feed treatment. Thus while some antibiotic was absorbed, much higher dose rates would be needed to achieve effective tissue levels, resulting in higher financial and environmental costs. Coated treatments are therefore not recommended. Tissue levels of oxytetracycline achieved in the same sized animals by as high-dose 2 hour bath treatment were also considerably lower than when milled into feed. Given the inherent wastage and environmental exposure by bath treatment, this method of administration is not recommended for this antibiotic (certainly for animals of this size).

During these trails, feed consumption, histopathology and haemocyte counts were used as indicators of antibiotic toxicity. As these antibiotics had been registered for use in other animals, and used at dose previously used successfully in other species, toxicity was not expected, though data showing safety to the treated animals was required. Interpretation of histological changes was confounded to some extent by the higher than expected level of tissue changes in control animals, although this is in line with studies in previous experimental systems and demonstrates the limitations of such systems. Oxytetracycline, the antibiotic of immediate concern, showed little overall tissue change after 5 days of oral treatment, though many showed an apparent mobilisation of haemocytes from the perivascular beds, and in some animals a slight increase, which could possibly represent a replenishment response. This is consistent with haemocyte counts undertaken during treatment trials, showing a diphasic circulating haemocyte response (a decrease, followed by an increase) with oxytetracycline treatment. It may be noteworthy that this antibiotic has also been shown to have an immuno-suppressive effect on fish. Of possibly more concern was the precipitates suggestive of calcium / divalent ion precipitation, which may indicate dying cells.

Changes suggesting acute tissue damage and increased renal excretion were more marked with the potentiated sulphonamide treatment. This suggests renal effects similar to those found in vertebrates with this treatment need to be considered if this antibiotic group is further considered in the future. Changes were slight with amoxicillin treatment, though one-day post treatment results suggested little antibiotic had been absorbed.

Objective 3: Coincidentally to increase understanding of factors affecting *V. harveyi* infections in abalone, and provide tools for further research into its control.

The tissue levels of residues reported in this study reflect a small number of tests from pooled animals due to the cost of analytical tests for antibiotics and limited budget for this indicative work. The commonly used practice of assessing antibiotic levels by bacterial growth inhibition was avoided due to concerns that such tests could be compromised by the reported

high levels of innate antibacterial factors in abalone. *In vitro* techniques to measure innate antibacterial activity were adapted in an effort to provide a practical method of differentiating innate and antibiotic related activity. The levels of innate activity proved too variable, both within and between groups, to provide a reliable assessment of antibiotic activity under the test conditions used. This work did provide some information on antibiotic levels, and considerable information on characteristics of innate factors and criteria for selection of the dilution range and indicator bacteria to monitor these factors. The combined results of innate resistance studies and experimental infection trials provided considerable information on the dynamics of susceptibility to *V. harveyi* infection.

The initial artificial infection trials demonstrated that quite large numbers of bacterial were required to establish lethal infections, though the level of resistance did vary between abalone batches. There was a narrow range between sub-lethal and overwhelming infections. In one trial animals tolerating bacterial infections of up to 10^6 well, with very few deaths, while almost all animals died (with or without antibiotic treatment) in a subsequent trial using a dose of 8 x 10^6 bacteria per animal. This is a constraint on experimental infections unless multiple dose trials are used as bacteria are administered at estimated dose rates, the actual dose being determined retrospectively.

Innate antibacterial factors were found to be measurable in a tube-dilation assay system, though sometimes highly variable, and to have a degree of specificity and be inducible after exposure to pathogenic bacteria. Thus inhibition of *V. harveyi* growth by exposed animals being much higher than inhibition of non-pathogenic bacteria from the abalone environment, indicating that indicator bacteria need to be the same or closely related to the bacteria of concern. Inhibition of this bacterial strain was negligible in naive animals tested in spring, but high (evident at dilutions up to 1:512) in animals which had been exposed to summer temperatures on farm and likely to have been exposed to this pathogen. These enhanced levels were apparently maintained for some weeks after movement from the farm, findings which suggest further research is warranted into whether vaccine-like exposure to relevant bacterial components is protective for the summer period. The inhibition of this bacteria was apparently due to bacteriostatic rather than bactericidal activity.

OUTCOMES ACHIEVED

Of the 5 antibiotics tested, only oxytetracycline was confirmed as being adequately absorbed and clinically active when milled into feed, with efficacy demonstrated against *V. harveyi*, the major pathogen of immediate concern. Absorption of this antibiotic from food was moderate, and residue clearance remains a concern. Absorption of all antibiotics from agar coated food was significantly lower than treatments milled into the feed. A relatively small Absorption of this antibiotic by bath Absorption of other antibiotics was poor, with the exception of bath absorption of oxolinic acid, which is no longer acceptable for food producing animals, and moderate levels of sulphadiazine from bath treatment. However as sulphonamides are unlikely to be used alone, and trimethoprim was ineffective in trials against *V. harveyi,* this provides little opportunity for use unless further work identifies a more useful potentiator.

Knowledge of the characteristics of *V. harveyi* infection and dynamics of innate bacterial factors was also gained.

KEY WORDS: abalone, antibiotics, oxytetracycline, amoxicillin, sulphonamide, oxolinic acid, trimethoprim,

Acknowledgments

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1 BACKGROUND

1.1 General Background

With the development of intensive abalone aquaculture in Australia, mainly as land-based pump ashore farms, episodes of mortality associated with bacterial infections emerged, often initiated by stress events which were out of abalone farmers' control. Infection of juvenile abalone with *Vibrio harveyi* has been described in at least two States following stresses such as high summer temperatures (Reuter and McOrist, 1999, Handlinger et al., 2002 & 2005). This was of particular concern because, unlike most *Vibrio spp.* infections that in general appear primarily septicaemic, these *V. harveyi* type infections resulted in multiple, poorly circumscribed abscesses that provided a continuous source for reinfection and recurrent mortality over subsequent months. This pattern of infection is similar to that reported in Japan (Nishimori et al., 1998) and more recently in France (Nicolas et al., 2002) as infection by *Vibrio carchariae*. Gauger and Gomez-Chiarri (2002) confirmed that *V. carchariae* is a junior synonym of *V. harveyi.*

This project arose from industry's desire to have antibiotic available for emergency use in abalone stocks experiencing high mortality, especially for *V. harveyi* type infections, where early antibiotic treatment appeared appropriate to prevent the establishment of chronic infections. Although off-label veterinary prescription of antibiotics for this use was legal, and had to some extent been used (with equivocal results), there was concern that such use was likely to become more restricted (JETACAR report, 1999), no antibiotics were registered for use in abalone, and their use may be impractical due to difficulty in application. Industry was mindful that appropriate data for antibiotic use was virtually absent for molluscs and very limited for other aquatic invertebrates, and that inappropriate use in abalone in other countries has resulted in multiple antibiotic resistance to abalone bacterial pathogens (Li et al., 1996).

Because of small potential markets, Australian pharmaceutical companies were unwilling to finance appropriate studies. As few developed countries culture molluscs intensively, no similar studies appeared to have been undertaken elsewhere, except one study of the efficacy of several antibiotics against the intracellular rickettsial agent of withering syndrome in Californian (Shields and Freidman, 1999).

The aim of this initial project was to gain an indication of whether antibiotics could be effectively administered to abalone in a practical manner, identification of the most promising antibiotics and an indication of whether antibiotic use was effective against bacterial infection. It was considered that further research to address all the parameters required for development of an application for registration or a Minor Use Permit (MUP) was not warranted unless there is an indication that sufficient stability, bioavailability, and tissue levels can be achieved. Such a decision would also be influenced by whether alternate approaches offer better potential for disease control

It was also important to obtain at least preliminary data on the rate of clearance of tissue levels, to ensure treated animals could legally be marketed in a realistic time-frame, as until sufficient data is presented to set a Minimum Residue Level of abalone, the legal tissue level is zero.

Analytical measurement of antibiotic levels is expensive. Consequently *in vitro* bacterial inhibition is favoured as an initial screening method for many antibiotic residue test programs, where possible. This further reduces the availability of analytical residue tests, given that standards require these to be validated in the species under test. There was concern that such

in vitro bacterial growth inhibition tests may be compromised by the high level of innate antibacterial factors which had long been reported in abalone (Li, 1960). Conversely, because of the costs of analytical measurement of antibiotic levels, *in vitro* assessment of bioactivity was considered desirable if this activity could be taken into account. It was considered likely that *in vitro* techniques development to measure innate and antibiotic related activity enable these two factors to be differentiated. This would enable *in vitro* assessment of antibiotic activity to be used as an adjunct to analytical methods, but would also provide information on the innate activity and its dynamics in abalone. The main laboratory development required to achieve this was a simplified, repeatable method for comparing the anti-bacterial activity in multiple samples of haemolymph.

Similarly, the need to develop a model of infection to assess *in vivo* activity would add to our knowledge of the determinants of susceptibility to infection, which may also aid in developing alternate means of disease control.

1.2 Project development

Following the expansion of the abalone aquaculture industry and the occurrence of farm-threatening bacterial diseases warranting antibiotic use, concern was raised by prescribing veterinarians in 1999 regarding the lack of registered drugs for use in abalone, and the lack of data on absorption, toxicity and chemical residues available to veterinarians. This was supported by all veterinarians and other diagnostic health professionals servicing the industry at that time.

At the invitation of the Steering Committee of the FRDC Abalone Sub-program, a pre-proposal for research to provide data for a National Registration Authority Minor Use Permit (MUP) application for use of one or more antibiotics in abalone was developed, but was considered by the Sub-program Steering Committee to be beyond current available resources, particularly with the uncertain outcome. This preliminary project was designed to investigate practicality aspects of administration and provide an indication of whether sufficient stability, bioavailability, and tissue residue levels can be achieved to justify future research.

1.3 General Introduction

Of the bacterial diseases reported to cause significant losses of farmed abalone, "blister diseases" caused by *Vibrio harveyi* (including bacteria previously described as *Vibrio 'carchariae'*, a junior synonym for *V. harveyi*) (Pedersen et al*.,* 1998; Gauger & Gomez-Chiarri, 2002) and *Vibrio fluvialis II* produce perhaps the most devastating acute outbreaks. Potential overall losses are increased by the tendency for the bacteria to become sequestrated in abscesses or pustules, which can become sources of further infections for prolonged or repeated outbreaks. Emergency antibiotic use under such circumstances has been attempted, with varying success, and in China with development of antibiotic resistance (Li et al., 1996). As currently no antibiotics are registered for use in abalone, and consequently no Minimum Residue Level (MRL) have been set, such practices also create significant implications for antibiotic residue control.

Development of a legal framework, such as registration or a Minor Use Permits, to enable an MRL to be set, and to allow continued use, requires significant input to provide the required data on efficacy and environmental safety. Pharmaceutical companies are reluctant to finance appropriate studies because of small potential markets size, especially while there remains doubt regarding the practicality of antibiotic administration. Factors affecting antibiotic use (reviewed by Treves-Brown, 2000), potentially include antibiotic instability or leaching during slow feeding, characteristic of abalone and reduced bioactivity of antibiotics in marine

invertebrate systems. There was doubt on continued legal availability of drugs, such as the quinolones, for use in food producing animals. In particular, available data (Treves-Brown, 2000), suggests markedly reduced bioactivity of antibiotics such as the tetracyclines and quinolones in a seawater environment, due to chelation with cations such as Ca^{++} and Mg^{++}. Chelation resulted in as little as 1-2% bioavailability even for pellet feeding animals, such as marine fish, where the overall contact with external marine ionic levels is much less than for slowly feeding abalone. Data on appropriate dose rates is virtually absent for molluscs. Abalone remains the only mollusc widely cultured under intensive systems which make potential antibiotic use an issue. Very limited data is available for antibiotic characteristics in other aquatic invertebrates.

In order to determine if any antibiotics warrant the significant cost to obtain registration, a series of preliminary investigations were undertaken to evaluate which antibiotics could be effectively administered in a bioactive form, given these constraints and the slow feeding pattern of abalone.

The choice of antibiotics to evaluate as potential candidates for registration was limited by the cost of obtaining environmental safety data that would meet registration authority requirements. This restricted the choice of antibiotics that are already at least partially accepted for other aquatic animals, and within accepted antibiotic groups to the antibiotic most widely used for aquaculture. The five antibiotics identified on this basis as most likely to be useful in abalone were the tetracyclines, of which oxytetracycline is the most widely used, amoxicillin, sulphonamides in combination with low doses of trimethoprim, trimethoprim used alone, and the quinolones, of which the most widely used in aquaculture in Australia is oxolinic acid. With the exception of trimethoprim alone, which was not initially assessed for absorption, each antibiotic was assessed using three administration methods (bath treatment, milled into artificial feed, and coated onto feed) in blacklip abalone (*Haliotis rubra* Leach) at normal summer temperatures for this species. As the primary objective was to verify absorption, dose rates were equivalent to or greater than the maximum reported use in other aquatic animals, taking into account suggested confounding factors. Higher levels were used where absorption failure was already strongly suspected. Tissue levels of antibiotic were measured at the end of the treatment period and one week later. Feed consumption, histopathology and haemocyte counts were used as indicators of antibiotic toxicity.

To test bioavailabiliy after absorption, both *in vitro* and *in vivo* approaches were used. *In vitro* measures based on haemolymph titration must address both the salinity conditions present in abalone tissues and innate antibacterial activity. Of particular note are the high levels of divalent ions such as calcium and magnesium ions, which are known to potentially chelate at high levels for some antibiotics, especially quinolones and the tetracyclines (Treves-Brown, 2000,). This author quotes studies suggesting seawater concentrations of MgCl may increase the MIC (minimum inhibitory concentration) of these antibiotics 40-60 fold. Information on the stability of these chelated compounds is therefore also important. Knowledge of the levels and dynamics of antibacterial factors in at risk abalone gained while validating *in vitro* antibiotic measurement was recognised as inherently useful to determine future directions for disease control research.

For these reasons, analytic methods of antibiotic measurement were used as the definitive measure of tissue antibiotic levels and samples were referred to laboratories registered for such tests by the National Association of Testing Authorities (NATA). As well as this, methods to undertake *in vitro* antibacterial activity were adapted with the duel aims of understanding of innate antibacterial activity dynamics, and *in vitro* antibiotic measurement if practical.

The latter required an initial step to develop methods to measure innate activity that were sufficiently reliable and repeatable to differentiate and compare antibiotic and innate antibacterial activity. Given the relatively small number of animals and limited resources, the usefulness of such tests also depended on variability of levels of innate activity within a group, as this will determine whether differences in bacterial suppression can be detected between animals given or not given antibiotics. Similarly, the variability of antibiotic absorption between animals will also affect the sensitivity of the comparison.

Simple means such as zones of inhibition are in general too inaccurate and insensitive for quantitative assessment, and haemolymph factors are likely to be too dilute to inhibit the density of bacteria which result from plate growth. Serial dilution techniques provide a quantitative measure of inhibition (for example, Hubert et al., 1996), though definitive, quantitative assessment requiring serial plate culture of dilutions near the inhibitory point increasing the work load per sample. To provide a test with a realistic work load, a variant of this method used previously in crustaceans to measure total antibacterial activity (Norton et al., 1999), was adapted. Although initial trials suggested non-pathogenic bacteria from the abalone environment would be suitable indicators, further tests to determine a suitable indicator and test conditions suggested the pathogenic abalone isolate identified as *V. harveyi* strain 01/0022 was more suitable. This isolate was eventually chosen as the preferred indicator, ensuring both improved sensitivity and relevance to the primary target disease. This was used for assessment of antibacterial activity from later exposure trials, though samples from initial antibiotic absorption trials were assessed against the earlier indicators.

Because of the uncertain reliability of *in vitro* measurement of retained bioactivity, some *in vivo* efficacy trials were required to validate the usefulness of the chosen antibiotics. Large scale mortality trials were beyond the scope of this work, but a small scale treatment trial was conducted to measure *in vivo* activity of the most promising antibiotic (oxytetracycline). As significant but not absolute mortality in untreated animals is a requirement for any treatment assessment (as antibiotics or other treatments are unlikely to be effective against overwhelming challenge), development of a standardised model of infection was attempted for this purpose. The aim was to determine a standardised dose, given by intra-sinus infection, of *Vibrio harveyi* strain 01/0022 recovered from naturally infected abalone, to provide an LD 50 or lethal dose to approximately 50% of the animals and thus suitable for efficacy assessment. However high levels of natural resistance, variability of response and very narrow dose-rate window between survival and death of all animals prompted opportunist use of a natural infection for this purpose.

2 NEED

Bacterial infections (especially *Vibrio harveyi*) had emerged in the abalone aquaculture industry as mortality episodes related to stress events that were considered largely are out of abalone farmers' control. Data for appropriate antibiotic use was unavailable. Inappropriate unregulated use in shellfish including abalone has led to antibiotic resistance and residue problems in several countries (Supriyadi and Rukyani, 1996; Cruz-Lacierda et al., 1996; Li et al., 1996). Legal antibiotic access in Australia was limited, and there was an expectation that this situation would worsen with proposed legislative changes (later summarised in the JETACAR report, Anon, 1999), and that in the longer term antibiotic use would require at least MUP registration. MUP would require data on a range of parameters, including tissue levels achieved with various dose rates and how this varies with size, species, temperature and physiological state, duration of residues in tissues, acute and longer term toxicity, and efficacy against the pathogens of concern under the proposed conditions.

Environmental safety data requirements would limit the choice of antibiotics to those already available for other aquatic animals, that is oxytetracycline, amoxicillin, potentiated sulphonamides (trimethoprim / sulphadiazine combination), and possibly quinolones such as oxolinic acid. Each of these has major potential limitations in either stability or bioavailability under marine conditions, efficacy against current pathogens, or acceptability for aquaculture use (quinolones). Volumes required for bath treatments, leaching from feeds, and cessation of feeding in infected abalone could all limit practicality.

Addressing all these parameters for MUP application was not warranted unless sufficient stability, bioavailability, and tissue levels are achievable, hence this preliminary study of the practicality of administration and *in vitro* and *in vivo* bioassay of bioactivity.

Industry appreciates that avoidance is preferable to treatment, and though stress may sometimes be unavoidable, understanding its role and being able to measure the effect may assist in developing alternate health management strategies. It was considered likely that development of the *in vitro* techniques to measure and differentiate innate and antibiotic related activity and a model of infection to assess *in vivo* activity may prove useful for future studies into the dynamics of bacterial infection and the possible role of stress in development of bacterial disease.

3 OBJECTIVES

4. To confirm the potential for antibiotic control of bacterial infections of abalone
5. To determine which antibiotics are most suitable for use in abalone aquaculture and appropriate mechanisms of delivery.
6. Coincidentally to increase understanding of factors affecting *V. harveyi* infections in abalone, and provide tools for further research into its control.

4 METHODS

4.1 Experimental design

The project was designed as a preliminary series of trials aimed at resolving the three major issues regarding antibiotic use in abalone. The first is whether a suitable delivery system can be provided for each antibiotic. The second is whether the antibiotic remains active within the abalone tissue. The third is the time for residues to be cleared to an acceptable level, so that the abalone can legally be marketed.

The first set of experiments were to test a range of antibiotics for the levels absorbed after administration at expected treatment levels by bath, milled into feed, and coated onto feed in agar. This set of experiments was to determine the most promising antibiotics for further work.

To avoid interference by innate antibacterial factors and keep within budget, antibiotic levels in a small number or samples were measured by chemical methods by standard accredited antibiotic testing laboratories. The aim was to also assess *in vitro* antibiotic activity in a system allowing comparison of total antibacterial activity in pooled samples from treated animals with the innate antibacterial activity in a control pool from the same group. A method was developed for measurement of *in vitro* activity by titration of haemolymph for inhibition of bacterial growth in a tube assay. This was used to assess antibiotic activity, with some results confirming the antibiotic retained biological activity, but overall innate antibacterial activity proved too variable for this use. Therefore only analytical means were used as a measure of antibiotic levels, though the study of innate antibacterial activity was valuable for increasing our knowledge of abalone / bacterial interaction, and as a preliminary study of methods for future research in this area. This trial also included an assessment of toxicity, based on the effect on survival, feed consumption, haemocyte counts and on tissue histology.

The second set of experiments were to demonstrate efficacy (effectiveness) in one or two chosen antibiotics against a common abalone bacterial pathogen (*V. harveyi)*, isolated from abalone cases. This required development of a standard and reproducible method of reproducing a suitable level of infection by careful control of artificial exposure. Well established criteria suggest overall mortality levels of approximately 50 – 70% are optimal for efficacy trial. If the infection level is too light, it is difficult to measure antibiotic effect as the abalone itself will likely self-cure. If too heavy, no level of antibiotic use is likely to prevent death. Thus the first step was preliminary experiments without antibiotics, to confirm an appropriate dose of *V. harveyi* for a useful level of infection. Stress as a component of the infection model was also considered as farm histories indicated that infection was often precipitated by stress. Antibiotic efficacy was then assessed against such an artificial infection. As it proved difficult to standardise the infection, this was then repeated using an opportune natural infection.

4.2 Antibiotic absorption trials

4.2.1 Antibiotic delivery systems

Antibiotics were administered by three methods, milled into feed, dissolved in an agar coating of feed pellets and as a 2.5 hour bath. The aim was to initially test absorption of a

representative from each of the antibiotic groups that had been used in aquatic animals, and for which some data existed. Dose rates were those estimated from use in other aquatic animals to be the maximum likely required dose if absorption patterns mimicked those of other marine animals. For comparison of relative absorption of different antibiotic groups, trials were run concurrently wherever possible. Bath trials were run separately from the feed trials, but for comparison of absorption from different administration methods, the bath treatments for each antibiotic group were run as close as practical to the feed trial for that antibiotic, using the same group of animals and same batch of antibiotic.

Due to the large number of tanks required, feed absorption trials for the 5 antibiotics were split into two trials run approximately 8 weeks apart, using the same cohort of abalone. Bath trials were run separately from the feed trials.

Animals were monitored during experiments, and after completion of each treatment trial, treated abalone and untreated controls were sampled for antibiotic residues, histological and microbial examinations, including the bioassay system for total inhibitory activity of haemolymph. Haemocyte counts were also undertaken where sufficient haemolymph was available. Additional animals were held for a further 7 days when samples were collected for antibiotic residues. Due to cost and the preliminary nature of this work, not all tissue samples were submitted for analysis. Primary testing was carried out on selected samples, others were held for further testing when the pattern of absorption or toxicity was unclear. Any mortality during the experimental period were also held, and analysed selectively.

4.2.2 Choice of antibiotics

The choice of antibiotics to evaluate as potential candidates for registration was limited by the cost of obtaining environmental safety data that would meet registration authority requirements. This restricted the choice of antibiotics that are already at least partially accepted for other aquatic animals, and within accepted antibiotic groups to the antibiotic most widely used for aquaculture. The five antibiotics identified on this basis as most likely to be useful in abalone were the tetracyclines, of which oxytetracycline is the most widely used, amoxicillin, sulphonamides in combination with low doses of trimethoprim, trimethoprim used alone, and the quinolones, of which the most widely used in aquaculture in Australia at that time was oxolinic acid. With the exception of trimethoprim alone, which was not initially assessed for absorption, each antibiotic was assessed using three administration methods (bath treatment, milled into artificial feed, and coated onto feed) in blacklip abalone (*Haliotis rubra* Leach) at normal summer temperatures for this species.

4.2.3 Choice of dose rate

As the primary objective was to verify absorption, dose rates were equivalent to or greater than the maximum reported use in other aquatic animals, taking into account suggested confounding factors: higher levels were used where absorption failure was already strongly suspected. There is a considerable body of reports on antibiotic use in fin-fish, but few of these are systematic studies. There is also considerable experience with antibiotic use in aquaculture which is not formally reported, especially in countries with little control on antibiotic use. Reports on the use in mollusc were almost absent. As well as specific reports quoted, major sources of information on dose rates used in other aquatic animals were the major review of Treves-Brown, 2002, with additional information on use patterns from Agenda Papers for the 1996 regional meeting on the use of chemicals in aquaculture in Asia (Anon, 1996). Tissue levels were measured at the end of the treatment period and one week later.

4.2.4 Experimental holding system

Abalone were obtained from a commercial abalone farm and maintained at low stocking densities in 60 l tanks in a recirculating seawater system for approximately 3-4 weeks prior to experimentation. To minimise stress during transport, abalone were carefully removed from substrate at source, transported in large plastic bags lined with moist foam, sealed with air or oxygen, and cooled during transport. Animals were placed in cool, oxygenated water as soon as possible, and lightly fed initially. Temperature was controlled to 16-17°C, close to spring-summer temperatures for this species. Temperature, salinity and dissolved oxygen (DO) were monitored to acceptable standards.

For reasons of cost (given the preliminary nature of the trials), recirculation was continued during experiments on antibiotic absorption from feed, but not during trials involving infection. Although this is a potentially confounding factor, calculated dilution rates indicate that the effect on antibiotic absorption is slight. Tanks were cleaned and food added 2-3 times per week.

4.2.5 Animals for absorption trials

Blacklip abalone (*H. rubra)* of approximately 30 to 55 mm in shell length and 25 g mean weight were obtained in five intakes from the commercial abalone farm (during the warmer months of November through to March) and held for a minimum of 3-4 weeks prior to experimentation, when weights and lengths were measured.

Duplicate tanks were used in all absorption experiments. A preliminary oxytetracycline absorption trial consisting of 10 animals / tank (2 tanks treated, 2 untreated controls), used a different cohort to the remaining trials. All other absorption trials consisted of 20 animal / tank with 2 tanks for controls and for each treatment. The same cohort of animals was used for all of the main antibiotic absorption trials where antibiotics were administered by three methods, milled into feed, dissolved in an agar coating of feed pellets and as a 2 hour bath. To the extent this was possible, feed based trials were run concurrently, in order that direct comparisons could be made between antibiotics and food administration method. The number of tanks required this to be split into two trials, each directly comparing absorption by both feed administration systems, with the oxolinic acid trial being carried out approximately 8 weeks after trial of the other antibiotics and the bath treatments. All bath trials were run concurrently, after the first of these trials, on the same cohort of animals.

4.2.6 Oral administration of antibiotics

Abalone diet was prepared as required from a commercial diet premix, supplied by Adam and Amos Pty. Ltd. 40% water was added to the premix and a commercial pasta maker (Italpast) used to produce the pellets. The pellets were dried overnight at 30°C, and refrigerated until use. Abalone were maintained on this diet for several weeks. Treatment diet was produced as above, with the inclusion of the antibiotic, either by milling into the feed at the time of batch preparation or dissolved in an agar coating of feed pellets. After the five day food treatment period, animals were returned to normal diet for 24 hours to purge the gut of the majority of unabsorbed antibiotic before sampling.

Antibiotic feeding levels were calculated retrospectively as the actual food under these conditions was unknown but variability in feeding rate under experimental conditions suggest this is likely to be less than the maximum 2% / body weight day previously recorded. Allowing for a probable reduced feeding rate, antibiotics were incorporated or coated onto food at a rate which at the estimated maximum food consumption rate of 2% body weight /

day would give the following daily dose rates: 200 mg /kg/day for oxytetracycline (OTC); 80 mg/kg/day for amoxicillin; 30 mg/kg/day for oxolinic acid (OXA); 30 mg/kg/day of total active ingredients for trimethoprim/sulphadiazine (TMP/SDZ); or 40 mg/kg/day of trimethoprim. (Inclusion rates of active ingredient at 1%, 0.4%, 0.15%, 0.15%, and 0.2% respectively).

Table 1. Dose rates for administration of antibiotics to abalone.

Chemical	Desired dose rate mg/kg abalone	Desired dose in daily food[1]	Activity	Antibiotic inclusion level (%)	Total dose g/kg food
Oxytetracycline	200	200mg in 20g	980mg/g	1.020	10.204
Amoxicillin	80	80mg in 20g	840mg/g	0.476	4.762
Oxolinic acid	30	30mg in 20g	998mg/g	0.150	1.503
TMP/Sulphadiazine	30	30mg in 20g	480mg/g	0.313	3.125
Trimethoprim (TMP)	40	20mg in 20g	1000mg/g	0.208	2.083

Abalone were fed the treatment diet at the estimated maximum feeding rate of 2% of body weight per day for 5 days, and actual food consumption measured. Uneaten food was collected twice during each experiment, once after 2 days, and again after 3 days, and dried overnight at 35°C to provide a measure of apparent food consumption. This was based on the overall food loss which includes food eaten by abalone, metabolised by microorganisms, leached by the water, and other particulate losses due to the messy feeding habits of the abalone. This method has provided reliable results for previous work with abalone (Harris et al., 1998).

4.2.6.1 Oral absorption experiment 1. Oxytetracycline

In a preliminary experiment, 10 abalone of mean length and weight 52.75±0.59 mm (mean±SE, n=40) and 20.17±0.63 g respectively were placed into each of four tanks (duplicate treatment and control tanks) and allowed 3 days to acclimatise to the tanks before experimentation began. OTC was incorporated into the food at the level listed in Table 1. Normal diet was fed to the abalone on day 5. 5 abalone from each tank were sampled for haemolymph and placed in formalin for histological examination, and 5 abalone were frozen for antibiotic residue analysis on day 6. On day 13, 5 more abalone were frozen for residue analysis.

4.2.6.2 Oral absorption experiment 2. Oxytetracycline, amoxicillin & trimethoprim/ sulphadiazine

Duplicate tanks for OTC, AMX, TMP/SDZ and control treatments, using two methods of delivery for each antibiotic treatment (in and on the prepared food), were stocked with abalone of mean length and weight 49.21±0.41 mm (mean±SE, n=186) and 18.53±0.41 g, respectively. The experiment was conducted in the same way as for Experiment 1.

4.2.6.3 Oral absorption experiment 3. Oxolinic acid & Trimethoprim

Duplicate tanks for OXO, TMP and control treatments, using two methods of delivery for each antibiotic treatment (in and on the prepared food), were stocked with abalone averaging 47.79±0.64 mm (mean±SE, n=89) and 16.76±0.61 g length and weight respectively, and

[1] Based on food consumed (g/kg abalone/day) @ 2% bw/day for 20 g abalone

rested as for Experiment 1. The experiment was conducted in the same way as for Experiment 1.

4.2.7 Bath administration of antibiotics

For bath treatments, abalone from the same cohort as for feed trials were transferred to treatment tanks 3-5 days before experimentation. As no chemical tests were available for trimethoprim, this antibiotic (as a stand-alone treatment) was not assessed. The 10 treatment tanks provided duplicate tanks of 15 abalone / tank for controls and 4 antibiotic treatments, enabling these to be run concurrently. On day of treatment, water level was lowered in each tank to 5 l, while aeration was maintained. The appropriate antibiotics were added to the treatment tanks (Table 2), left for 2 hours, then rinsed and refilled from normal water supply. Longer baths were not considered likely to be a practical option for abalone farms. Groups of five abalone from each tank were immediately sampled for haemolymph and histological analysis, and for antibiotic residues. Water quality including salinity and temperature were monitored and corrected where necessary to maintained between 29.15 and 29.9 ppt, temperature between 14.0 and 14.3. The tanks were maintained as described in 4.2.4, then 7 days later a further 5 abalone were sampled from each tank for residue analysis, and frozen with original residue samples.

Dose rates of active ingredient used to measure absorption from bath treatment in seawater were 1000 mg/l for oxytetracycline, 400 mg/l for amoxicillin, 150 mg/l for oxolinic acid, 150 mg/l for TMP/SDZ. Abalone weights were 18.53±0.41 g for the mixture of oxytetracycline, amoxicillin and trimethoprim / sulphadiazine.

Table 2. Dose application rates for antibiotic bath treatment

Chemical	Desired dose rate mg/kg abalone	Activity	Antibiotic inclusion level (%)	Amount added per 5 l
Oxytetracycline	200	980mg/g	1.020	5.1g
Oxolinic acid	30	998mg/g	0.150	0.75g
Amoxicillin	80	840mg/g	0.476	2.4g
TMP/Sulphadiazine	30	480mg/g	0.313	1.6g

4.2.8 Samples from absorption trials

After completion of the bath treatment or 24 hour purge of medicated feed, soft tissues from five abalone from each tank were frozen for antibiotic residues. A further five animals per tank were assessed for possible toxic effects of antibiotic treatment by clinical examination, and haemolymph counts and / or histological examination. Haemolymph (HL) samples were removed from the cephalic sinus via 2.5 ml syringe and 25 gauge needle, with haemocyte counts being undertaken where sufficient haemolymph was available. Remaining animals were maintained as normal for a further 7 days, when 5 abalone were similarly sampled from each tank for residue analysis.

A the rate of residue decline from tissues should be independent of administration method and of tissue levels (at least near the active range), 7 day residues after oral administration were measured only from abalone consuming antibiotics milled into the feed, which were expected to provide the highest initial levels. (Samples from other tanks were collected and held frozen if required.)

4.2.9 Chemical measurement of antibiotic residues

Pools of frozen samples were submitted to accredited antibiotic testing laboratories (State Chemistry Laboratory, Victoria, and Australian Government Analytical Laboratories, N.S.W.) for chemical analysis for antibiotics.

Samples for amoxicillin, oxytetracycline and sulphonamide (sulphadiazine) were submitted to State Chemistry Laboratory, Victoria, and were analysed according to their test codes 20250/20254, 20155, and 20158 respectively.

Oxolinic acid samples were submitted to the Australian Government Analytical Laboratories, N.S.W. (AGAL), for analysis using an LC/MS/MS method. Initial analysis used the method . developed for oxolinic acid and similar antibacterial compounds in rainbow trout. As this was not fully validated for abalone, standard procedures included abalone blanks and abalone blanks spiked with oxolinic acid with each test batch (pers. comm. B. Woodward, AGAL, 2001). Later analysis was done after validation for abalone by replicate extractions at three spiking levels, with the average method recovery for oxolinic acid found to be 65% (range 50 – 60%), with nalidixic acid as a surrogate recoveries for the batch ranging from 37 to 53% (pers. comm. L. Johnson, AGAL).

To minimise cost only selected samples were submitted, the remainder being held for later submission if required. The rationale for selective testing was that the rate of residue decline is normally independent of the initial tissue level (within the normal biologically active range). Thus in the feed administration trails, day 7 samples were generally measured only from the in-feed treatment as this was likely to provide higher tissue levels than with feed coating, where leaching was an anticipated constraint.

4.3 Assessment of antibiotic toxicity

Feed consumption, histopathology and to a lesser extent haemocyte counts were used as indicators of antibiotic toxicity. Haemocyte counts were performed on fresh haemolymph samples using a haemocytometer with Nebauer ruling, which was filled immediately after collection. Counts were made as soon as cells had settled. For histopathology, abalone were then fixed in 10% seawater formalin, following which cross sections to include all major organs were processed through paraffin, cut and stained by haematoxylin and eosin by routine methods. Slides were read blind with regard to treatment, with each tissue change being described and scored for severity between 0 (no change) and 3.

4.4 Assessment of antibacterial activity *in vitro*

The recognition that innate antibacterial factors in haemolymph would affect any *in vitro* measurement of antibiotic inhibitory activity necessitated that the initial phase of this work was the development of methods for a quantitative assay that could compare bacterial growth inhibition due to innate antibacterial factors alone to inhibition resulting from both innate and antibiotic activity.

Development of a suitable test required adaptation of simple quantitative methods for measuring the anti-bacterial activity from those used for crustaceans by Norton et al., 1999, taking into account the expected lower overall levels of innate soluble antibacterial activity in crustaceans (Soderhall, 2001). Methods based on growth of bacteria in serial dilution of haemolymph in culture media were used as these provide a quantitative assessment. Qualitative and semi-quantitative assessment using inhibition of bacterial growth on plate culture were used for verification purposes. Bacterial colony counts after incubation in haemolymph were used to verify qualitative assessment of growth in the serial dilutions. The

method was later modified when a greater range of activity and interference from natural (sub-clinical) bacterial carriage within the haemolymph was found during the summer months.

Anti-bacterial activity was assessed *in vitro* by measuring the dilution of haemolymph which inhibited 24 hour bacterial growth or survival. This used serial dilutions of haemolymph and a standard level of indicator bacteria (*V. harveyi* in the later trials) in a tube test system, followed by plate culture to assess bacterial viability. A number of trials were undertaken to establish suitable test criteria prior to and in parallel with comparison of total antibacterial activity of antibiotic and untreated abalone. Thus samples from the early antibiotic trials were tested using a different indicator bacteria, providing a test of lower sensitivity.

4.4.1 Development of a quantitative assay for innate antibacterial factors.

4.4.1.1 Initial screen for suitable indicator bacteria

The initial criteria for selection of an indicator bacteria was for *Vibrio* strains that were sensitive to abalone haemolymph and preferably resistant to the test antibiotics.

As factors present in naive animals appeared not to inhibit the growth of pathogenic strains of *Vibrio harveyi*, other *Vibrio* species from the abalone environment were screened for inhibition by normal abalone haemolymph. Inhibition was assessed after selective culture on TCBS for *Vibrio* species isolated from the water and foam fractionator from the recirculation system, 2 weeks after abalone were added to the system.

A presumptive test for suitability was sensitivity to the vibriostat 0129, which is regarded as a defining characteristic of *Vibrio* species. *Vibrio* isolates were then screened for sensitivity to haemolymph and from the resident abalone, using lawn plates of *Vibrio* nutrient Agar (VNA) medium[1] with blank antibiotic sensitivity paper discs, impregnated with either 10µl of either neat haemolymph, lightly or heavily centrifuged haemolymph.

4.4.1.2 Quantitative *in vitro* assessment trial 1

The two bacterial isolates sensitive to abalone haemolymph from the initial broad scale recirculating seawater system screening (termed FF1 and Q1) were then prepared as a bacterial suspension of known density (90 NTU; approx. 5 x 10^8 cells/ml) with Mueller-Hinton broth[2] (MHB). A replicated serial dilution of haemolymph was incubated with standard amounts of a bacterial suspension. This was carried out in microtitre trays. After 24 hours incubation all wells were assessed for apparent growth, rated as light medium or heavy. Samples from each microtitre well were then inoculated onto *Vibrio* nutrient Agar (VNA) plates in a qualitative test to confirm growth.

This test was conducted using 2 abalone, and set up with 2 bacterial isolates, according to the set-up and protocol in Tables 3 and 4. Because of the limited quantities of haemolymph available, only one abalone was titrated against each bacterium.

[1] Derived from West, P. A. & Colwell, R.R. 1984. Formulation details in Appendix 3.
[2] CM0405, Oxoid, Adelaide

Table 3. Outline of microtitre plate row set up for assessment trial 1.

Row	Dilution	Recipe
A	1:1.1	10 µl (200 µl of 50 NTU in 1800 µl MHB) + 90 µl HL
B	1:2	50 µl (0.5 NTU) + 50 µl HL
C	1:4	50 µl (0.5 NTU) + 50 µl 1:2 HL
D	1:8	50 µl (0.5 NTU) + 50 µl 1:4 HL
E	-ve control	50 µl MHB + 50 µl HL
F	+ve control	50 µl (0.5 NTU)
G	Haemolymph control	100 µl HL

(MHB = Muller-Hinton Broth, HL = Haemolymph), NTU = Nephelometric Turbidity Unit

Table 4. Protocol for columns on abalone haemolymph tests in microtitre trays for test 1.

	Abalone 1			Abalone 2		
	Isolate FF1			Isolate Q1		
No.	1	2	3	4	5	6

4.4.1.3 Quantitative *in vitro* assessment trial 2

The second test was set up with triplicate tubes of haemolymph dilutions, with haemolymph from each abalone titrated against both bacteria, (Table 5). Later *in vitro* trials utilised a similar protocol, with full details provided in Appendix 3.

Table 5. Protocol for columns on abalone haemolymph tests in microtitre trays for test 2.

1	2	3	4	5	6	7	8	9	10	11	12
Isolate FF1						Isolate Q1					
Abalone 1			Abalone 2			Abalone 1			Abalone 2		

4.4.1.4 Experiment 3: Timed kill of bacteria by normal abalone haemolymph

This test provides a quantitative measure of bacterial survival following a 4 hours incubation of a bacterial culture with haemolymph. For this trial only Isolate FF1 was used. The haemolymph was sampled at the beginning and end of a 4 hours incubation period by serial dilution and incubation on plates.

For this experiment 15 ml of Muller-Hinton broth (MHB) was distributed to 15 ml turbidity tubes. Sufficient bacteria were added until 50 NTU was reached. Of this, 50 µl is added to 4950 µl of MHB, giving a dilution of 1:100. 15 µl of this suspension was distributed to each well of rows A and B. Haemolymph (HL) was removed from abalone via cephalic sinus (2.5 ml syringe, 25 gauge needle), 1.2 ml being required. Raw haemolymph (120 µl) was added to each well of rows A (test samples) and C (negative controls), 15 µl of MHB to each well of row A, 135 µl to each well of row B (positive controls), and 30 µl to each well of row C. (Table 6) At this stage haemolymph was not filtered.

The procedure was repeated with a second pair of sera, using triplicate plates for each well.

Table 6. Outline of microtitre plate row set up for time-related survival index.

Row	Dilution	Recipe
A	10:1 HL to bacteria	15 µl (bacterial suspension) + 120 µl HL + 15 µl MHB
B	+ve control	15 µl (bacterial suspension) + 135 µl MHB
C	-ve control	120 µl HL + 30 µl MHB

(MHB = Muller-Hinton Broth, HL = Haemolymph)

Table 7. Protocol for columns on abalone haemolymph tests in microtitre trays, using timed culture.

1	2	3	4	5	6	7	8	9	10	11	12
Isolate FF1											
Abalone 1						Abalone 2					
Time 0			Time t (4 h)			Time 0			Time t (4 h)		

All plates were incubated at 25° for 3-4 h. The actual number of viable bacteria were determined at the onset of the experiment by aseptically removing 100 µl aliquot's from each well, serially diluted through two tenfold dilutions in MHB and plated in triplicate onto VNA plates. Further samples were taken at 4 h and similarly plated. All plates were then incubated at 20°C for 48-72 h until distinct colonies could be distinguished. The number of colony forming units (CFU's) was determined for each plate by direct counting. This method provide data to determine a survival index (SI), expressed as SI = (cfu at time (t))/(cfu at time (0)) x 100.

4.4.1.5 Experiment 4: The effect of filtration and incubation temperature

The effect of longer in-well incubation, the effect of filtration and different incubation temperatures were assessed after it was found from the previous experiments (which were run in January) that variability of the *in vitro* test response increased with the use of abalone exposed to summer temperatures prior to transfer to the laboratory system. The tests for the next experiments were run at both 15°C, and 25°C, followed by confirmation of visual assessment of growth in well plates, but with an extended dilution series (undiluted to 1:32) compared to the previous experiments.

In an attempt to increase the sensitivity, two incubation temperatures were compared, well plates were assessed at 24 hours incubation and again after 48 hours to ensure sufficient bacterial generations to assess growth*, Vibrio harveyi* isolate 2201 was used as an indicator, and the dilution series extended to 1:32. This was extended further in succeeding experiments to 1:512. Microtitre plate set-up as for Table 3, except for the extended dilution series (Full details in Appendix 3.)

In order to remove and evaluate confounding bacteria, either from natural carriage or contamination during collection, both filtered and non-filtered (0.2µm syringe filtration) haemolymph were used. Filtration was done immediately, from the syringe used to collect the sample, therefore only minimal products derived from ruptured haemocytes would be expected (unless cells are ruptured during this process). This plus the small volumes collected from abalone of this size precluded a direct comparison of filtration using haemolymph from the same abalone.

Potential for antibiotic use in abalone

In order to keep within a realistic workload, results were assessed primarily by visual assessment of growth in wells. For this experiment these results were confirmed with a qualitative plate streak on VNA plates after 48 hours.

All of the filtered sample wells were plated onto VNA after 48 hours.

4.4.1.6 Experiment 5: Heat-challenged abalone

A second (additional) experiment was undertaken on this cohort to further assess extending the haemolymph dilution range (undiluted to 1: 512), utilising haemolymph from animals used for a heat-challenge trial undertaken as a general assessment of haemocyte responses and reported previously (Handlinger et al., 2004). Abalone were from the same cohort as the previous experiments, and held at 15°C for several weeks before transfer to water at 15-16°C or 21°C. Over the trial period of 6 days, 11/20 animals in the higher temperature group and 2/20 animals in the lower temperature group died.

The haemolymph from four of the eight control abalone was removed via syringe and 25 gauge needle from the cephalic sinus. These samples were incubated at 15°C and form an extended replicate of *in vitro* Experiment 3.

The remaining abalone for this experiment were bled from the base of the foot, via the removal of a small cube of the foot muscle. This was because only small abalone were available and filtration of haemolymph was to be used to prevent confounding of results by contaminants.

For all plates 0.2 µm filtered haemolymph was used. Plates from foot-bled abalone were again incubated at 15 and 25°C.

4.4.1.7 Experiment 6: Effect of heat inactivation on innate antibacterial activity

This experiment was conducted to determine any effects between fresh haemolymph and heat-inactivated haemolymph, again at 15°C and 25°C.

The experiment was set up as for the previous experiment. Samples from four abalone were filtered and held in a water bath at 56°C for 30 minutes. All twelve haemolymph samples were serially diluted and 50 µl aliquot's transferred to the microtitre trays. Two of the heat treated samples and two of the fresh samples were incubated at 15°C, while another tray of samples was set up the same and incubated at 25°C. Four previously frozen, filtered haemolymph samples were also serially diluted and prepared on a microtitre tray and incubated at 15°C.

4.4.2 Assessment of antibiotic activity *in vitro*

As well as analytical assessment of tissue residues, a preliminary *in vitro* assessment of antibiotic activity was also undertaken using the above methods, by comparing the total bacterial suppression in treated and untreated abalone, using the above trials as a guide to expected activity at the time of these trials.

For food absorption trials 1 (oxytetracycline only), and 2 (oxytetracycline, Amoxicillin and Trimethoprim/Sulphadiazine mixture), plus the bath experiment for these antibiotics, inhibition was assessed on serial dilutions to a maximum dilution of 1:8. A portion of haemolymph samples were filtered through 0.2 µm syringe filters and used for titration of inhibitory effect of growth of *Vibrio harveyi* (strain 01/0022) isolated from hybrid abalone and of a *Vibrio* sp. isolated from the abalone culture system (FF1). Dilutions of bacterial suspension commended at 10:1, then 1:2, haemolymph:bacteria, with doubling dilutions

thereafter, plus positive and negative controls. All tests were prepared in triplicate in Muller-Hinton Broth (MHB) with 1% salt, and both isolates were used for each sample.

For food administration trial 3 (oxolinic acid and Trimethoprim alone), the dilution range was extended to 1:512.

4.5 Assessment of antibiotic efficacy *in vivo*

4.5.1 Development of experimental infection model

To determine an appropriate injectable dose of bacteria to provide an infection lethal to approximately 50% of the animals and thus suitable for efficacy assessment, group of six abalone held at 16°C were first injected into the cephalic sinus with 100 µl of 10 fold dilutions of bacteria within the range of $10^2 – 10^5$ cells/ml (10-10^4 cells/abalone) and compared with injections of sterile seawater. In a second similar trial, bacterial concentrations of $10^5 – 10^9$ ($10^4 – 10^8$ cells / abalone) were injected 24 hours after raising the temperature to 21°C. Miles & Misra plate counts (Miles *et al.,* 1938) were used to determine the actual cell densities in the inoculum.

Any mortalities were retained in formalin for later examination. All remaining abalone were sampled on day 6. Haemolymph was examined for haemocyte counts and plated onto TCBS plates to check that the injected bacteria was present and abalone tissue retained in formalin to verify the presence of typical *V. harveyi* lesions (Handlinger et al., 2003), and to characterise the nature of the infection.

4.5.2 Antibiotic treatment of artificial infection

On the basis of results from the absorption trials and past on-farm use, oxytetracycline was chosen as the most likely candidate for continued assessment and clinical use. *Vibrio harveyi* 01/0022 was used for the challenge. Minimum inhibitory concentration (MIC) of OTC for this strain was carried out prior to experimentation, and determined to be 0.5 µg/ml in the presence of 1% salt, and 1.56 µg/ml in the presence of added divalent ions calcium and magnesium.

Abalone of mean length and weight of 50.0±1.0mm and 18.19±0.94g, respectively (mean±SE), were randomly assigned to 12 tanks to provide 6 treatments in duplicate 24 hours before 4 tanks commenced the OTC diet, as above. The following day, temperatures were increased to 21°C in all but two untreated tanks, which were held at 17°C. Two days later abalone in 2 OTC fed tanks and two untreated tanks were injected with *V. harveyi* (at an estimated dose of between 10^6 and 10^7 cells/ injection) and two untreated tanks with blank sterile seawater. The remaining tanks at 21°C and tanks at 17°C received no treatment. Miles & Misra plate count revealed the injected bacterial suspension to contain 8.2 x 10^6 cells / abalone. At this point, 10 abalone fed medicated diet but not injected with bacteria were sampled and frozen for antibiotic residue analysis. Mortalities were recorded over the next 7 days, at which time all surviving abalone were sampled for haemolymph and histological analysis. Uneaten diet was collected on two occasions and dried at 40°C to provide an estimate of apparent food consumption between medicated and unmedicated treatments. Temperature, salinity and pH were measured each day using a TPS WP80 handheld meter and combination pH electrode, and combination salinity/conductivity electrode. Salinity in tanks at 21°C varied between 33.6 and 34.3 ppt, tanks at 17°C slightly lower at 32.1 ppt, suggesting slight evaporation at the higher temperature. pH was between 8.14 and 8.27 in all tanks.

Potential for antibiotic use in abalone

For *in vitro* tests on abalone haemolymph from artificial challenge trial, haemolymph samples were filtered using 0.2 µm syringe filters and frozen until later use. The protocol was similar to the previous *in vitro* antibiotic assessments: 50 µl of haemolymph from one abalone was added to 50µl of Muller-Hinton Broth (MHB) with 1% salt in a microtitre tray, then serially diluted in MHB to a two fold dilution series from 1:1 to 1 in 512. An 82 NTU solution in MHB was made using a plate culture of *V. harveyi* prepared the previous day. This solution was diluted 1 in 100 and 50 µl aliquot's added to the wells containing the 8 serial dilutions and a positive control well. 10 µl of this bacterial solution and 90 µl of HL were added to the last well in the row (haemolymph control, providing an additional 1 in 10 dilution). Dilution series were prepared in duplicate for all samples. All preparation was carried out in a bacteriology extraction cupboard, and all trays were tape-sealed and incubated for 48 hours at 15°C.

4.5.3 Antibiotic treatment of natural infection

Animals for the second treatment trial were not brought into the controlled temperature system until February, by which time they had been subjected to summer stress conditions. Following the stress of transport and subsequent failures of temperature regulation in the experimental facilities, a natural outbreak of *V. harveyi* infection occurred in these animals. The nature of this infection was considered more directly relevant than artificial infection, overcoming much of the constraints of that system. Consequently the natural infection was used to test the efficacy of oxytetracycline, using the same dose as in previous trials.

Because this removed the necessity for multiple bacterial doses, there were sufficient tanks available to also test the efficacy of trimethoprim as a stand-alone treatment. Sulphonamide was demonstrated to be inadequately absorbed from food, though moderately well absorbed from bath treatment and potentially useful in this form if the potentiator, trimethoprim, was also absorbed. Trimethoprim (unlike the sulphonamides) is also a realistic treatment alone at higher levels. The initial absorption experiments had not provided a measure of trimethoprim absorption, due to a lack of a suitable analytical method for assessment in abalone tissues. This did not prevent a primary assessment of the absorption of the potentiated sulphonamide treatment the sulphonamide component is regarded as a better routine residue indicator for the mixture, given the low levels of TMZ used.

No disease outbreaks had been reported on-farm, but abalone had been subjected to summer temperatures when the 430 *H. rubra* abalone of approximately 50 mm length were transported from a commercial farm to the experimental facilities in February. Previous experience at several farms suggested such temperatures could be sufficiently stressful to precipitate outbreaks. Abalone were distributed between 18 tanks supplied by a common recirculation system, for a post-transfer acclimatisation period prior to the anticipated artificial treatment trial. Deaths were first noticed on day 5 post-transfer. On the follow days temperature control, which had maintained a temperature between 16 – 17^{o}C, dropped to 14^{o}C and fluctuated between 11-14^{o}C throughout the following day before repair. Deaths remained at high levels for at least 10 days but were declining by day 18 post-transfer when treatment was commenced. On that day two abalone were sampled for baseline data before abalone were re-distributed between tanks to provide 10 abalone / tank (60 abalone / treatment). The tank system was switched to a static water system with frequent partial water changes, and treatment with antibiotic feed commenced. Six tanks were randomly allocated to each treatment group (untreated, oxytetracycline or trimethoprim treated feed). Temperature control was less even after tanks were switched to a static water system with a peak temperature of 25^{o}C occurring on day 4 of the treatment period.

Potential for antibiotic use in abalone

All feeds were made fresh from the same base commercial diet premix, as described above and at the same inclusion levels. Uneaten food was removed daily. Tanks were examined and mortalities examined and removed at least daily.

Treatment was continued for 10 days (until Mar 7), when all groups were sampled (10-12 animals from each treatment) all tanks were switched to untreated food, and the system returned to recirculation. Sampling included haemocyte counts and bacterial culture on haemolymph, fixing of selected samples for histological verification of typical lesions, and sampling for antibiotic residues. As adequate whole body OTC levels had been verified previously for this dose rate, only pooled muscle tissue was analysed for OTC levels at the end of treatment. OTC level were measured in both gut and foot muscle from abalone dying within one day of treatment, as an indication of whether such animals were still feeding. After the remaining abalone were returned to untreated food, mortalities were monitored for a further 15 days, after which time a further sample of 12 animals from each treatment group were similarly examined.

5 RESULTS AND DISCUSSION

5.1 Antibiotic absorption trials

5.1.1 Oral absorption experiment 1. Oxytetracycline (from in-feed treatment)

For this preliminary experiment using OTC milled into feed, total soft tissue OTC levels of 78 and 9.5 mg OTC/kg (ppm) were recorded from pooled samples of 5 abalone collected 1 day and 7 days post-experiment, respectively.

Food consumption differed little between control and medicated abalone, but were considerably less than the estimated maximum (0.85 and 0.86 % bodyweight / day respectively), to give an effective feeding rate of 86 mg OTC/kg/day.

The minimum inhibitory concentration (MIC) of oxytetracycline for pathogenic *V. harveyi* (strain 01/0022) using media with 1% NaCl was 0.5 µg/ml. (That is, bacterial growth was inhibited at 0.5 µg/ml oxytetracycline, with growth occurring at 0.25 µg/ml.) In the presence of Ca^{++} and Mg^{++} the MIC level rose to 1.56µg/ml.

5.1.2 Oral absorption experiment 2. Oxytetracycline, amoxicillin & trimethoprim/ sulphadiazine milled into and coated on feed

5.1.2.1 Food consumption and effective dose rate

Food consumption was also less than maximum in this experiment where absorption of OTC, amoxicillin and trimethoprim/sulphadiazine (measured as the sulphonamide content) milled into or coated onto food with agar were assessed concurrently (Figure 1). Consumption varied with both diet and method of medication. There was considerable between tank variation even in the controls, where average feeding rates over 5 days were 1.1% and 2.2% / day. The agar coating alone appeared to contribute to lower feeding rates, as the rate of food intake was lower for all coated treatments compared to the same in-food treatment (including the controls), with the exception of amoxicillin, where a sharp decline in intake of the in-feed treatment was seen towards the latter part of the treatment period. A late reduction in feeding was also found for food coated with OTC and TMP/SDZ, though the antibiotic milled-in feed was consumed at a similar rate to the controls. The lowest feeding rate was for coated OTC, with an overall daily feeding rate was 0.48%, falling in both tanks in the latter part of treatment to an average of 0.36%/day. Overall the effect on food consumption was significant with both treatment ($p<0.001$) and method of application (on or in the diets) ($p<0.05$).

5.1.2.2 Antibiotic tissue levels: oxytetracycline

For OTC, the day 1 post treatment levels were 26, and 4.2 mg OTC/kg for milled into feed and medication-coated diet respective. The 7 days post treatment level for milled-in feed was 16 mg OTC/kg (Figure 2). As the overall food consumption rates for the milled-in and coated feed were 1.3% and 0.48% respectively, the estimated actual average daily dose rates were 130 and 48 mg/kg bodyweight/day (unadjusted for leaching).

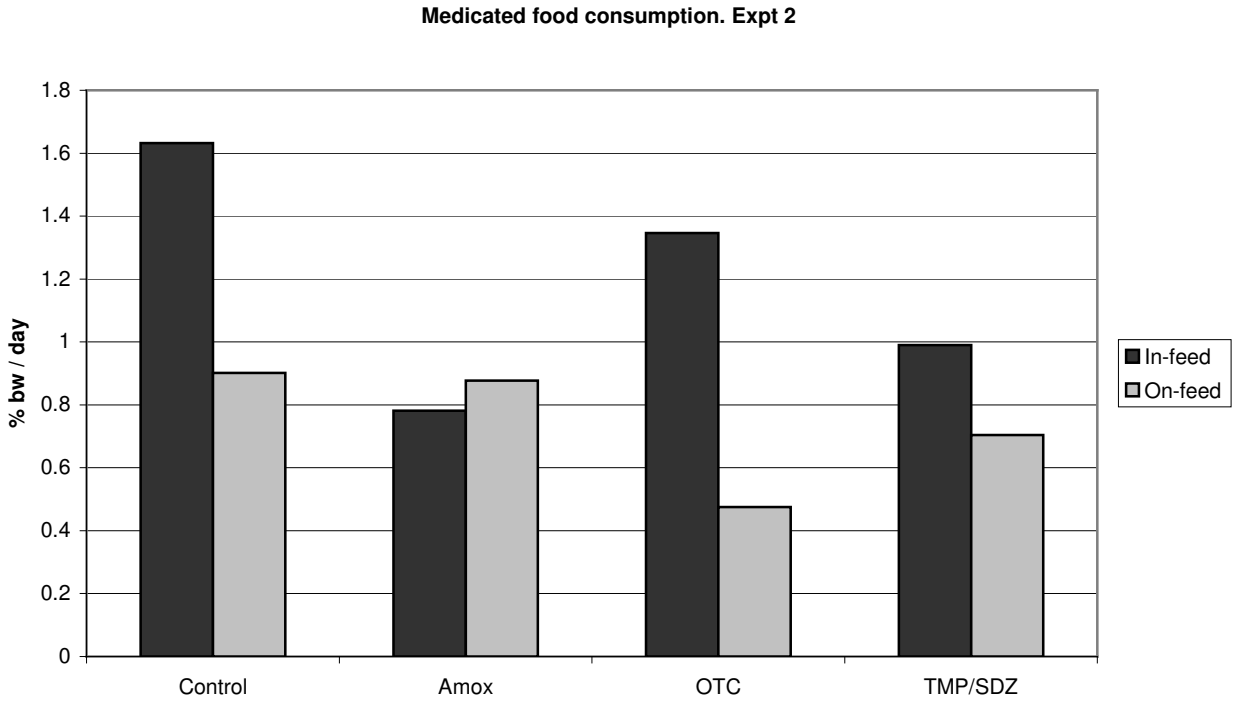

Figure 1. Effect of antibiotics and agar coating on food consumption.

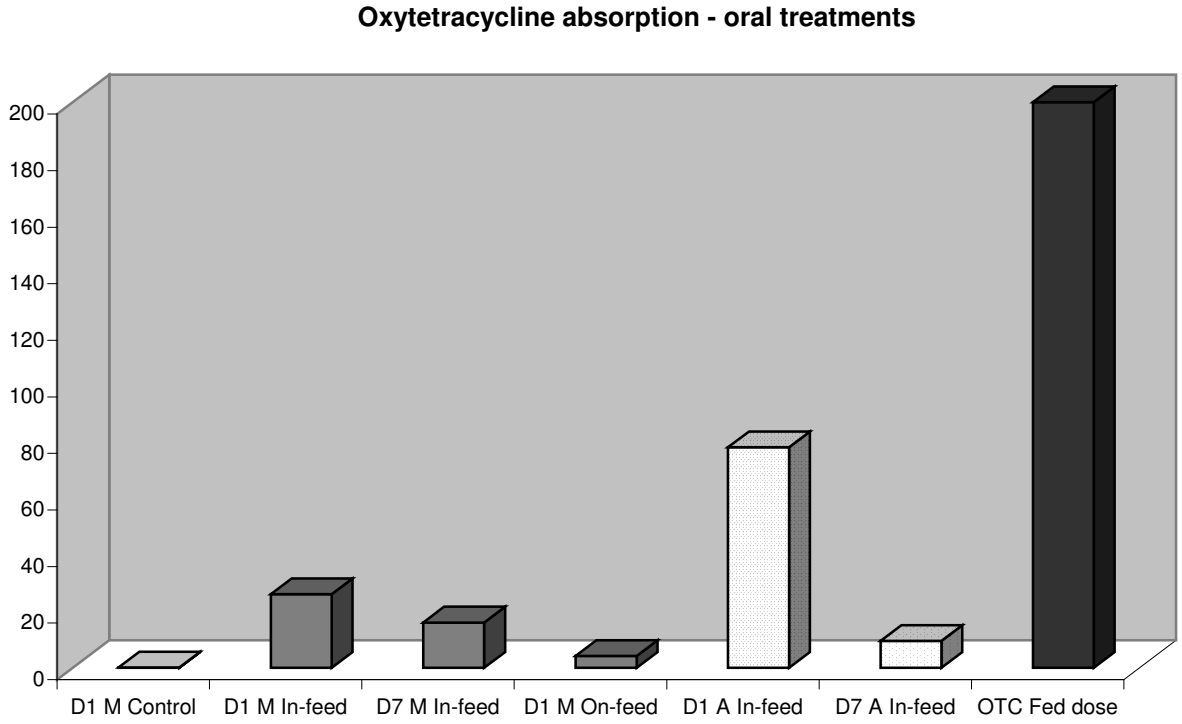

Figure 2. Relative oral absorption of oxytetracycline on days 1 and 7 post treatment from experiments 1 (pale) and combined treatment trial.

5.1.2.3 Antibiotic tissue levels: Amoxicillin

Amoxicillin levels in tissues from the two in-feed treatment tanks were 0.25 and 0.07 mg/kg on the first collection. No detectable levels of amoxicillin were found 7 days following this treatment, or from the day 1 samples from the on-feed pellet coated amoxicillin treatment (Figure 3). Overall consumption rates were average for the experiment and similar for the two tanks (0.83 and 0.73% bodyweight/day), giving apparent average consumed doses of 33 and 29 mg/kg bodyweight/day.

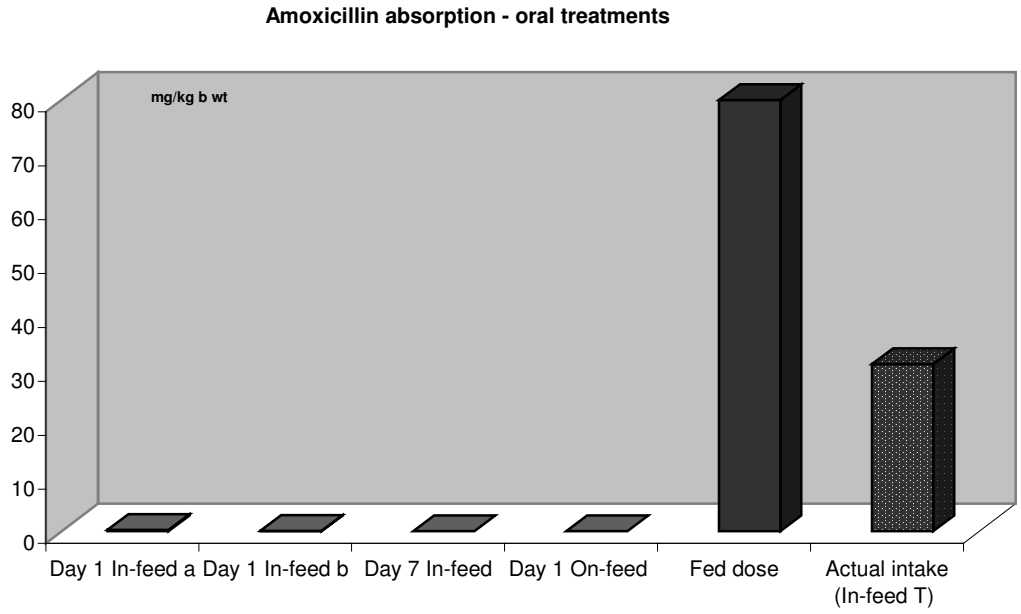

Figure 3. Absorption of amoxicillin from oral treatments.

5.1.2.4 Antibiotic tissue levels: potentiated sulphonamide treatments

Sulphonamide levels from the potentiated sulphonamide treatments from day 1 post treatment where 0.05 and 0.04 mg sulphadiazine/kg and for in-feed and medication-coated diet respectively, and 0.02 mg sulphonamide/kg at 7 days after in-feed treatment. Overall daily feeding rate for in-feed treatment was 1%, giving an actual in-feed treatment dose of 15 mg/kg total active ingredients (Figure 4).

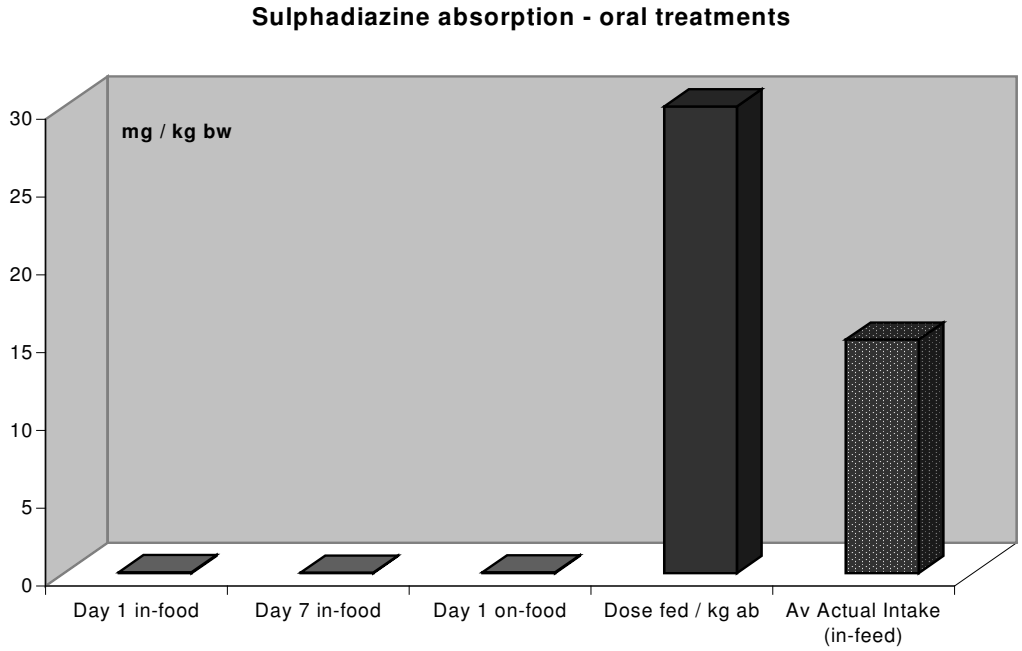

Figure 4. Absorption of sulphonamide component of oral TMP-SDZ combination treatments.

5.1.3 Oral absorption experiment 3. Oxolinic acid & trimethoprim

5.1.3.1 Food consumption and effective dose rate

Food consumption again varied with both treatment (p<0.001) and method of application (p<0.05) (Figure 5). In contrast to Experiment 2, the antibiotics had a greater effect on palatability in-feed than with the coating (p<0.05). More of the diets containing trimethoprim were consumed than the diets containing oxolinic acid (p<0.05). Because of variability in the controls consumption at rates different to the control abalone was not proven (p>0.05).

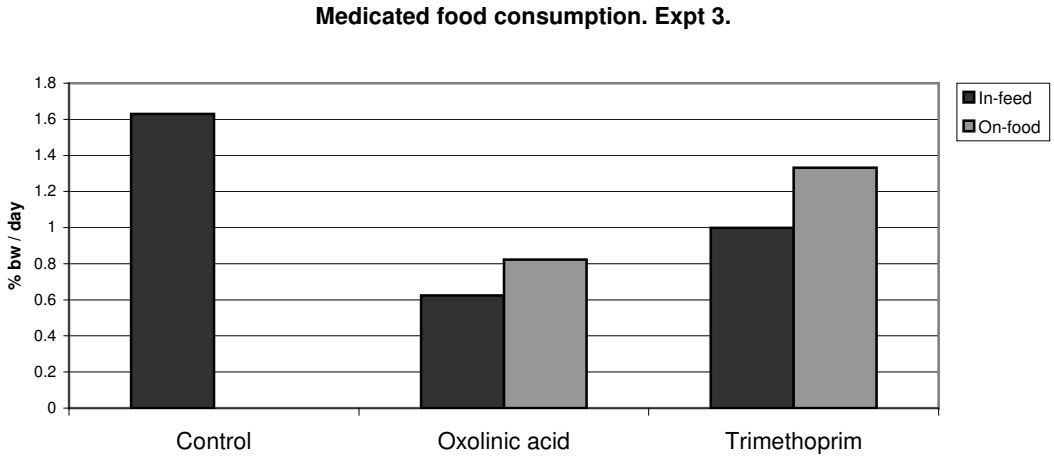

Figure 5. Effect of oxolinic acid, trimethoprim and agar coating on food consumption.

5.1.3.2 Antibiotic tissue levels: oxolinic acid

Overall daily feeding rates for in-feed and coated treatments were 0.8 and 0.6% bodyweight respectively: actual dose rates therefore being 12 and 9 mg/kg/day.

Potential for antibiotic use in abalone

Levels of oxolinic acid levels (OXA) after oral treatment were very low, regardless of method of presentation (Figure 6). The 24-hour (post purge) total body levels were close to or similar to residual levels 7 days later. Duplicate pools showed 90 and 105 µg OXA/kg for day 1 samples from in-feed treatment with residues of 65 µg /kg and 85 µg 7 days later. Coated feed treatment gave a day 1 pooled levels of 60 µg /kg with similar levels after 7 days. (Compare with tissue levels after bath treatment of 57 mg/kg).

5.1.3.3 Antibiotic tissue levels: trimethoprim

Tissue concentrations of trimethoprim (TMZ) were not measured as a validated assay methods for abalone flesh was not available during the period of these trials. Residues of this antibiotic are generally detected using biological assessment through bacterial inhibition, which was not used in these trials due to possible confounding factors arising from innate inhibitory factors in haemolymph. TMZ did not significantly reduce intake, either in-feed or as a coating, with feeding rates of 1% and 1.3% respectively (Figure 5)

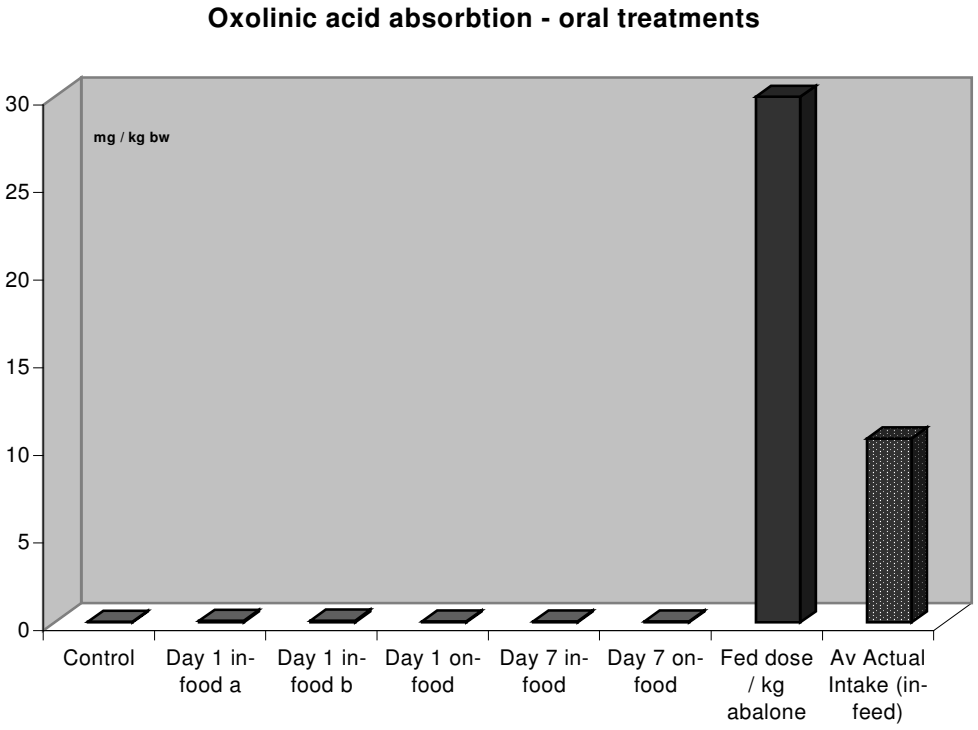

Figure 6. Absorption of oxolinic acid from oral treatments.

5.1.4 Antibiotic absorption from 2 hour bath treatment

The effect of antibiotic treatment in the experimental system on water is shown in Table 8, indicating that oxytetracycline and oxolinic acid required adjustment to maintain the desired levels.

Table 8. Water quality during antibiotic bath exposure experiment

Treatment	Salinity (ppt)	pH	Temperature (°C)
Amoxicillin	29.9	7.87	13.95
Control	29.8	7.99	14.1
Oxytetracycline	29.8	8.16	14.6
TMP/SDZ	29.15	7.23	14.25
Oxolinic acid	33.7	8.14	16.9

At this dose regimen OTC was absorbed less readily in bath exposure than from the in-feed treatment but better than from coated feed, with day 1 levels reaching 6.8 mg OTC/kg. Amoxicillin levels were 1.8 mg/kg, considerably more than the post-purge levels from food. The sulphadiazine component of the sulphadiazine/ trimethoprim mixture was also absorbed better than from the oral treatments, with a day 1 level of 2.9 mg SDZ/kg suggesting this may be a suitable mode of treatment if the trimethoprim component was also well absorbed and active. OXA was absorbed in abalone much more strongly than for food-medicated delivery, with levels of OXA following a 2 hour bath reaching 57 mg/kg. Antibiotic tissue levels achieved by bath administration, relative to those from feed, are summarised in Figures 7 –10.

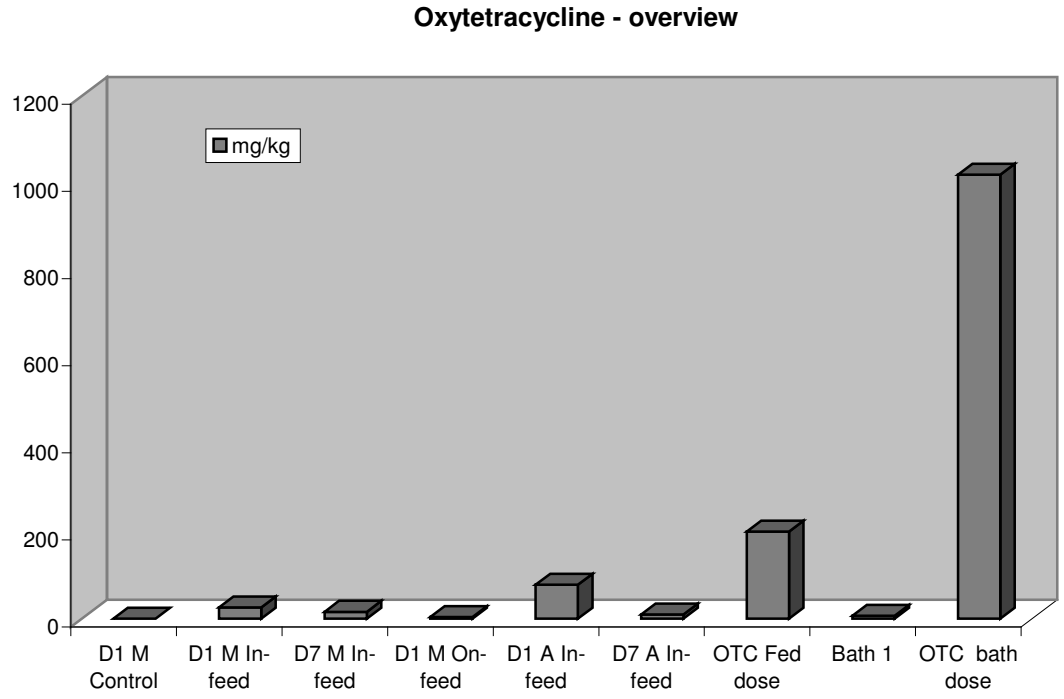

Figure 7. Comparison of oxytetracycline tissue levels with different treatments

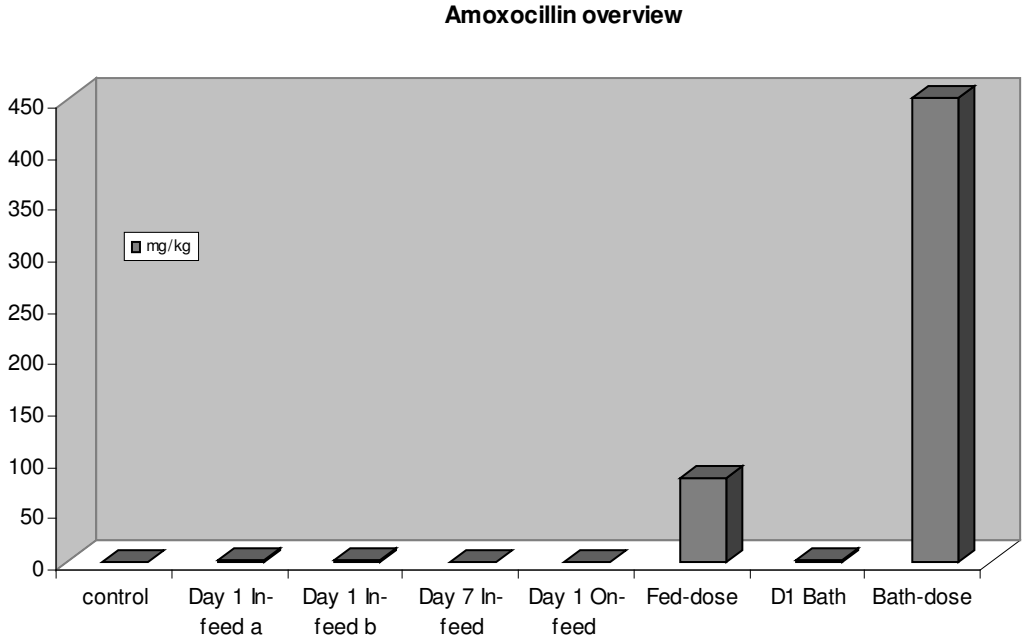

Figure 8. Comparison of amoxicillin tissue levels with different treatments

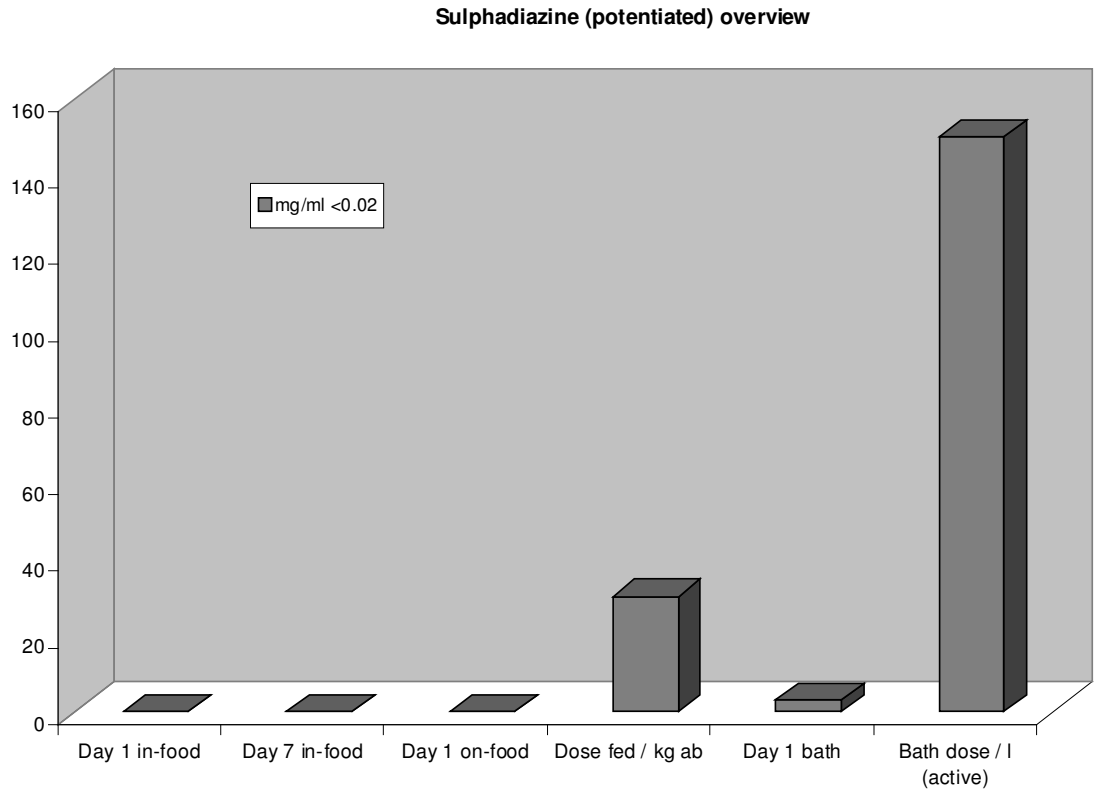

Figure 9. Comparison of sulphadiazine tissue levels with different treatments

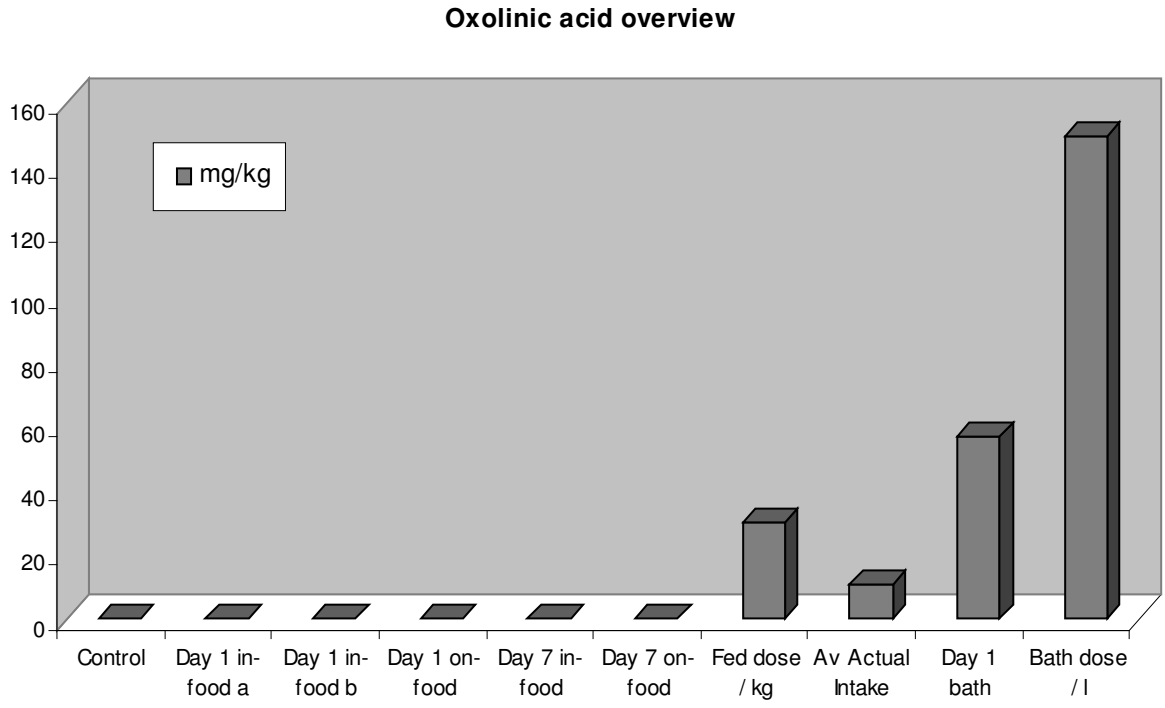

Figure 10. Comparison of oxolinic acid tissue levels with different treatments

5.1.5 Discussion

Oxytetracycline, the antibiotic so far most commonly used, was already known from field residue information to be well absorbed by abalone from food and only slowly excreted, but performance for disease control had been uncertain. The tetracyclines have low octanol/water partition coefficients and variable solubilities in water, but are soluble in both acids and alkalis. All are chelating agents, with complexes so formed being microbiologically inert and because they are electrically charged, cannot readily cross lipid-rich biological membranes. (Treves-Brown, 2000 p64) The high levels of chelation results in very low bioavailability when in contact with seawater (in some fish as low as 2% from feed), and may also be low without stomach acidification (only 0.6% oral bioavailability from carp). They will also complex with organic material (especially proteins) and clay. These antibiotics are hardly metabolised at all by fish.

Their activity against a broad spectrum of bacteria is their chief advantage. Disadvantages are prolonged residue times and that widespread use has already led to development of bacterial resistance in many countries. Thus, the worst case scenario for this antibiotic is that it is absorbed but most is inactive or pathogenic bacteria have developed resistance to it.

The usual recommended oral OTC dose rate for fish, and the maximum allowed by registration in several countries is 35-75 mg/kg fish/day, with the higher levels especially in marine fish, which in fish has been has high as 150 mg/kg. A high dose rate was used in light of previous variable field efficacy results and because of the high cation levels in seawater and longer contact time with a seawater-isotonic environment. The actual dose rate achieved with an inclusion rate of 1% active ingredient (10 g OTC/kg food), as calculated from apparent food consumption (85 and 130 mg/kg/day respectively) are only moderately above the recommended fish dose. Much higher dose rates of up to 42.22g OTC/kg feed dry weight, fed at slightly lower feeding rates, has been used successfully for experimental control of withering syndrome of red abalone (Friedman et al., 2003), reducing but not eliminating infection. However they also concluded that lower dose rates were likely to be effective, provided they could be administered evenly to all abalone in the tank. It should also be noted that as the causal rickettsial bacteria of withering syndrome is an intracellular rickettsia, effective tissue levels may well be higher than for freely circulating *V. harveyi.*

Comparison with fish suggest that absorption of OTC from milled-in feed at the inclusion rate used here with moderate food consumption is adequate for all tissues, at least for *Vibrio* infections. The full-body tissue levels of OTC achieved from in-feed treatment (78 and 26 mg/kg of bodyweight respectively in the first two trials), were in the order of maximum levels achieved in the liver of salmonids at optimum temperatures for absorption, such as liver levels of up to 46 µg/ml (= mg/kg) in trout, though levels in marine rainbow trout may be only 30% of those found in trout in freshwater. However they are considerably higher than effective levels in other fish tissues such as muscle, where typical maximum concentrations (C_{max}) are within the range of 2-4 µg/ml. Whole body levels had been measured as this is the standard tissue for mollusc residue analysis under Australian law, the whole body generally being eaten for most mollusc and for some abalone products. Foot muscle levels of 3.1 mg /kg (µg/ml from the final trial was within the range for fish muscle at a similar effective dose rate, suggesting that that adequate levels were achieved in this, perhaps the major target organ for *V. harveyi* infections. Using standard criteria for MIC measurement, these levels are above the recommended minimum tissue levels of 3x the MIC for typical sensitive bacteria such as the *V. harveyi* strain used for bacterial challenge (MIC 0.5 $\mu g.ml^{-1}$ OTC). However while whole body levels achieve the recommended 3x the MIC after feeding, foot muscle levels from *in vivo* trial 2 are marginal when assessed against the MIC in the presence of divalent ions,

which increased the MIC 3 fold (to 1.56µg/ml). Treves-Brown (p56) concluded from his review that the effect of these cations, especially magnesium, is concentration dependent, with *in vitro* MIC measurements increasing up to 50 fold following the addition of 50mMol magnesium to the agar. This is approximately the concentration in seawater (54mMol Mg^{++} at full salinity 35 ppt), and as molluscs do not regulate this ion, approximately the same concentration as mollusc haemolymph. Whether this is modified by other ions is unclear. Thus it was uncertain from absorption trials alone whether effective antibiotic activity was achieved with these methods of application, warranting further *in vitro* and *in vivo* trials.

Considerably higher levels were achieved for foot muscle (generally > 20 µg/ g) by Freidman et al*.,* (2003), using dosage up to 42.22g OTC/kg feed dry weight. The authors concluded that those dose rate may be excessive, even for treatment of withering syndrome of red abalone, though the requirement for this purpose may be greater than for *Vibrio* infections due to the intracellular nature of the rickettsial pathogen.

The rate of decline indicated by 7 day post treatment levels (9.5 and 16 mg/kg respectively) also varied between the two trials. Animals in the first trial appear overall to be biologically more active, the rate of decline suggesting a significantly shorter half life, so that residue clearance from the second trial may actually take longer, despite the lower levels achieved.

While all agar coated feeds appeared unpalatable to some degree, apparent consumption for OTC coated food was only 37% of the in-feed treatment, tissue levels at the end of the treatment period were only 16% of those achieved with OTC milled into the feed, indicating a considerable loss due to leaching and / or increased chelation. Thus this method of administration would appear to offer little opportunity for use with OTC for abalone.

OTC dose rates recommended for immersion vary from 5-120mg/l for freshwater, depending on hardness, but these are concentrations at which it would be expected to prevent the transfer of water borne infections but would not be therapeutic. The tendency for chelation suggests much higher levels could be required for seawater. Previous trials with abalone have used up to 400mg/L, with little success for disease control. (P. Hardy-Smith, pers. comm.). The level of 1000 mg/l was therefore regarded as maximal, though the actual tissue levels achieved (6.8 mg/kg) were actually higher than with the coated feed, though considerably less than in-feed treatment.

Amoxicillin is one of two semi-synthetic forms of penicillin which have a broad range of activity than the predominantly Gram positive spectrum of natural penicillin. Despite the overall improvement in activity against gram negative bacteria, activity is generally poor in the case of *Vibrio* species. This antibiotic was included to complete a broad overview of options for abalone treatment, rather than a likely treatment for *V. harveyi* specifically. These compound are weak acids, chemically rather unstable being decomposed by heat, oxidising and reducing agents, and rendered inactive by heavy metals and alkaline conditions. They are sparingly soluble in water, though some of their salts are more soluble, but do not form complexes with divalent cations and hence are relatively more useful in seawater than freshwater. Like oxytetracycline, leaching from coated pellets, both with oil and to a lesser extent agar coating, has been demonstrated previously (Duis et al., 1995).

Of the semi-synthetic penicillins, amoxicillin is more widely used in aquaculture, at recommended in-feed dose rates for fish of 40-80 mg/kg/day, for 5-10 days depending on the pathogen. The apparent dose rate achieved of 29-33 mg/kg/day is close to this range, though food intake was less than expected. Treves-Brown (2000) reports numerous cases of successful use but few reports of dose titrations in fish. Average C_{max} levels in serum, even following injection, appear low, ranging from 3.3 to 0.4 (µg/ml), and are not maintained for long periods. Because of the high solubility in water and recognised rapid excretion rate, it is

uncertain if the between tank variation in the whole body amoxicillin levels taken 24 hours after removal of food (0.25 and 0.07 mg/kg), actually represent an adequate tissue level or low levels of residual gut contents. However the high solubility suggests that a high level of leaching prior to intake may also be a factor. So too may be decomposition of the antibiotic before ingestion. (Treves-Brown, 2000, p.84) indicated that as much as 20% of amoxicillin may be lost through photo-decomposition when surface coated onto pellets.

Although this antibiotic is less likely to be useful for *Vibrio* infections than other species, further work may be needed to more accurately track tissue levels, or to demonstrate efficacy *in vivo,* for assessment against sensitive bacteria. It is also possible that the variability is a result of recognised problems with caking of this hygroscopic antibiotic, which may prevent it being evenly distributed. Though these compounds do not form complexes with divalent cations, absorption from bath administration was also very low, despite a high dose rate.

The sulphonamides are a large group of chemically related synthetic compounds, all derivatives of sulphanilamide. Most are only moderately soluble, though many are more soluble in seawater than freshwater. When used alone achieving effective tissue levels may be difficult, though the solubility of the sodium salt of any one sulphonamide is usually independent of the presence of others so higher levels can be achieved with a mixture of sulphonamides, and there is little margin between therapeutic and toxic levels. Resistance readily develops, and is common to all members of the group. Typical dose rates when used alone for fish are 100 – 200 mg/kg/day for up to 14 days, giving tissue levels in the order of 150-200 mg/kg.

Efficacy with much lower rates of use can be achieved by combination of a sulphonamide with trimethoprim, a pyrimidine analog, which can act as a potentiator. The combination is synergistic, because the components act as competitive inhibitors in the synthesis of folate, a precursor used for bacterial nucleic acid synthesis. The combination delays, but does not entirely prevent, the development of bacterial resistance because the bacteria have to produce resistance factors for the two drugs simultaneously. Ideally the pyrimidine analog and sulphonamide should have the same pharmacodynamic profile, but this is rarely tested. Of the seven effective diaminopyrimidines, only 3 are used in veterinary medicine and only two, trimethoprim and ormetoprim have been used in fish. The choice of combinations is usually pragmatic, on the basis of availability, and the combination of sulphadiazine (SDZ) and trimethoprim (TMP), marketed as a premix for use in fish (Tribrissen® 40% Powder), has been shown to be a useful veterinary preparation in a wide range of applications. The normally recommended dose regimen for this combination in fish is 30 mg/kg total active ingredients daily for 7-10 days, which represents 15% of the standard rate for sulphonamides. Typically the MIC for this combination is 0.2 – 0.8 µg/ml for the fish pathogen *V. anguillarum*, compared to MICs for SDZ and TMP alone of approximately 6 and 1.6 µg/ml respectively. Though the food level was calculated to achieve the fish recommended dose of 30 mg/kg sulphadiazine, the actual consumed dose was half this level. However the whole body tissue levels of the sulphonamide component achieved from medicated food were only about one tenth of those estimated to meet the expected MIC levels and therefore clearly inadequate for treatment.

The sulphadiazine component of the sulphadiazine/ trimethoprim mixture was absorbed better for bath treatment, with a day 1 level of 2.9 mg SDZ/kg suggesting this may be a suitable mode of treatment if the trimethoprim component was also well absorbed and active.

TMZ absorption by abalone was not directly measured, but has been shown to occur in trout in seawater bath uptake studies. Using a bath dose of 75mg/l TMP, which is close to saturation (Bergjo and Sognen, 1980), the uptake of trimethoprim by the adapted fish achieved tissue levels of 1 ppm in 10 hours and up to 5 ppm over an 84 hour exposure period. These

levels should prove sufficient for the combination therapy, but suggest TMP is unlikely to provide 3x the MIC level when used alone since for *V. harveyi* strain 01/0022, the MIC for trimethoprim alone was 1.56µg/ml and in the presence of Ca^{++} and Mg^{++} ions the MIC was 6.25µg/ml. The bath dose of 150 mg/l TMP/SDZ (in the ratio of 1:5) would provide a water level of TMZ close to half saturation, suggesting adequate absorption could occur and may be worth further investigation.

The quinolones, or more strictly the 4-quinolones, are a related group of synthetic carboxylic acid antibacterial agents which inhibit the bacterial enzyme DNA-gyrase. This enzyme heals DNA threads after they have been 'nicked' to allow folding, and inhibition results in unrepaired breaks of the DNA. The effect is generally bactericidal. Only a limited number of quinolones have been studied in fish. Of these, oxolinic (a true 4-quinolone) was developed specifically for fish and has been most widely used in fish.

Quinolones are amphoteric, most are sparingly soluble at neutral pH, but the sodium salts are soluble and absorbed through gills. They form bacteriologically inactive complexes with divalent cations, particularly Mg^{++}. There is a linear relationship between the minimum bactericidal concentration (MBC) and ionic concentrations in the range of 0.3 – 14.5 nMol Mg^{++}. Seawater contains 54 mMol Mg^{++}. It is likely that gut contents and possibly other tissues of marine animals will complex with quinolones given in feed. Bioavailability varies with dose rate, species and type of preparation (eg 14% to 35% for trout, higher for superfine product). Absorption and activity are both temperature dependent. For oxolinic acid the standard oral recommendation for fish was 10 mg/kg/day in freshwater species (similar to the level actually consumed by the abalone), but higher doses may be required in marine species, with regulatory limits varying between countries in the range of 25-50 mg/kg/day. Except at cold temperatures, typical C_{max} for OXA for fish is approximately 2 µg/ml for plasma and 4 µg/ml for skin, muscle and kidney. The oxolinic acid tissue levels achieved from medicated food were approximately 2-5% of these levels. As excretion is relatively slow (eg a terminal half-life of 42 hours following oral administration in Atlantic salmon), it is very unlikely that this represents rapid excretion. Indeed this is confirmed by the small decline in residues after 7 days.

In contrast OXA was absorbed in abalone much more strongly from bath than for food-medicated delivery, with levels of OXA following a 2 hour bath reaching 57 mg / kg. This was much higher than expected, though it is well recognised that quinolones may be actively taken up from water, potentially reaching higher levels in tissues than the surrounding water. Both the dose rate (150 mg/l) and findings are similar to those of Samuelsen and Lunestad (1996) in halibut, who found levels of up to 73 ppm for abdominal organs and 9.4 ppm for muscle following a 72 hour bath of 200 mg/l for vibriosis. They also noted this was higher than normally achieved by oral administration and considered that effective levels were likely to be maintained for several days following treatment. Dose rates reported for bath administration of fish are very variable, but do show active uptake. Uptake by goldfish, for example, has been shown with a dose rate of 10mg/l for 72 hours, though shorter times are likely to be more practical. However for practical reasons bath administration of any antibiotic is likely to be suitable only under selected circumstances, due to the large volumes required. These findings suggested that OXA could be administered effectively by bath treatment, in situations where this was practical but that lower dose rate would provide effective tissue levels. However there was always doubt about the long term availability of oxolinic acid for food producing animals, and withdrawal for these purposes has now been confirmed (Anon., 2003)

In summary, the absorption trials suggest practical administration can be achieved with oral treatment of OTC, bath treatment of OXA, possibly bath treatment with potentiated

sulphonamides if trimethoprim is also absorbed. There is some uncertainty regarding amoxicillin due to the relative speed of clearance of gut contents and antibiotic residues. Prolonged residue clearance for OTC limits the situations where OTC treatment is appropriate.

Although trimethoprim met the criteria for inclusion and absorption tests were done, validated analytical tests were not available and therefore tissue levels were not measured. The above results suggested that knowledge of absorption patterns could be of value for the use of this antibiotic either alone, or in combination with a sulphonamide for bath treatment. A knowledge of efficacy would also be required. There was opportunity to test efficacy *in vivo.* The apparent dose rate of 1% would have provided an average daily intake of 20 mg/kg bodyweight, which is similar to dose rates previously used for cutaneous erosion disease in Atlantic salmon caused by *Tenacibaculum maritimum*, though rates of 5mg/kg bodyweight/day are more common (Cameron, 1992). The efficacy trial against a natural *V. harveyi* infection clearly showed that TMZ absorbed from medicated feed was either inadequate, inactive, or the bacteria were resistant (see section 5.3.3). Therefore there was little value to pursue validation of analytical methods for measurement of trimethoprim residues for oral administration for use against *V. harveyi* infection. As effective levels of TMZ appear unlikely to be absorbed by a short bath for use as a sole treatment, only bath absorption of trimethoprim for use in combination with sulphonamide appears likely to warrant further assessment. However the suitability of absorption for pathogens more sensitive for this antibiotic muse be regarded as untested.

5.2 Histological assessment for antibiotic toxicity

As safety as well as efficacy is a requirement for antibiotic use or registration, a total of 175 abalone were examined histologically to provide an indication of toxicity, recognizing the limitations of assessing this from only a single dose rate per antibiotics per administration method, chosen to be at or near normal clinical activity. Samples from oral absorption trials were fixed one day after the completion of the 5-day treatment period, those from the bath treatment samples shortly after the completion of the 2 hour bath and return to normal seawater. No samples were collected from the second bath trial.

Not all tissues were recovered in section from each animal. This is frequently the case with small organs such as the left kidney. Budget constraints prohibited re-cutting for other tissues, though material has been held for this if required. Results of the tissue change scores for each change for each organ are summarised in Table 9. For this table, results for administration in the food, or coated onto the food have been pooled. A descriptive summary per treatment, per group is given below.

5.2.1 Histological effects after oral absorption

Oral absorption experiment 1: Effect of oxytetracycline

In the 9 controls examined from the preliminary oxytetracycline absorption trial, the only abnormalities were a little protein deposition in left kidney of 4, and slightly dilated lumens of right kidneys. Perigut haemocyte stores were abundant. One showed increased kidney pigment (possibly an older change). The slight kidney changes are likely to be related to short-term pump failure during the experiment.

The 11 OTC treated animals examined showed more foot surface lesions. Such changes resembled artefacts at time of sampling, but could possibly reflect a real change in behaviour or mucus characteristics that affected adherence. One showed slight non-fibrillar protein foci in the left kidney, otherwise changes were similar in range and extent to controls.

Oral absorption experiment 2:

Controls: Controls from the first combined feed trial were more variable, with no apparent differences between the 9 animals receiving normal food and the 10 fed antibiotic/agar-coated food. Approximately half of the animals showed reduced gut filling and / or poor overall condition as indicated by increased tissue spaces round the gut tubules and dilation of other interstitial spaces. Minor foot blisters were present in 2 of those fed normal food and 8 of those fed antibiotic/agar coated food, some gills were slightly thickened, and two animals showed small bacterial reactions under the gut epithelium. One dilated pericardium accompanied by slight grey deposit (possibly calcium) was seen in the coated food group.

Oxytetracycline: Three of the 7 abalone fed OTC in the diet showed bacterial infections, with early abscess formation in the foot of 2 of these. Other changes noted such as contracting protein in gill vessels plus pericardial dilation in the milder affected animal, and a major gill vessel clot plus focal myocarditis in another, plus a blue fibrillar appearance of the left kidneys appear related to the infection rather than the medication. The 10 animals fed the OTC coated diet showed no overt infection and no additional changes except for slight foot blisters and bluing of associated tissue in two. The peri-gut vascular bed was increased in two and showed variable dilation in others.

OTC from coated-diet: Of the 10 animals examined from the OTC coated food group, mild blisters were seen in four of the 6 feet examined. Six left kidneys were seen in section, 3 with slight fibrillar protein pooling. Five animals showed slightly dilated right kidney tubules. One animal showed moderate protein in the right kidney, plus protein the heart and oedema round the gut (left kidney not in section). Thick peri-gut bed shown by 2 animals, one with slight foot blisters and bluing of foot vessels plus some old protein precipitates, and dilated right kidney. (No foot section of other animal, but heart was dilated.). Other animals were relatively normal except some dilation of the peri-gut granulocyte layer as well as peri-tubule spaces (overall poor condition, plus vascular bed dilation).

Amoxicillin: The 10 animals fed amoxicillin in the diet and 9 fed amoxicillin coated food showed overall similar changes to the controls. In the animals given the antibiotic in-feed, these included slight foot loss (4 animals), mild blister like dilations (in 7), slight peri-tubule spaces and variable increase kidney pigment (indicative of poor condition, poor feeding rate and probable recent mild catabolism), and some showed an empty gut. Three showed a little protein in the left kidney, one of these with a slight fibrillar appearance, one mild protein plus some bluing and cell accumulation, and two with moderate protein. The perigut granulocyte bed was abundant in two, and variably slightly dilated. In those receiving amoxicillin coated food, food intake and condition was also. They showed slight foot abrasions and blisters, one with associated sub-epithelial protein pooling and one of three left kidneys examined showed very slight fibrillar protein. The peri-gut haemocyte bed was increased in three animals. The other animals showed haemocyte reactions in the mantle near base of the gill, with occasional bacteria.

Trimethoprim/ sulphonamide mixture: Of the 8 animals fed the potentiated sulphonamide mixture incorporated into the feed, 7 showed some blistering of the foot muscle. Grey to blue discolouring fine precipitate was associated with the blisters in 5 animals. Three also showed mild epithelial loss. Haemocyte pooling was seen in the left kidney of 5 animals (ranging from marked in 2 to mild), plus protein precipitates in 4 kidneys (one severe, one moderate, 2 mild). In addition to the left kidney pooling, protein pooling or precipitation was also present in 3 right kidneys, 2 gills and 2 hearts from these 4 animals. Three right kidneys showed dilated tubules, one of these with tubule thinning. Renal tubule pigment varied from light to heavy in three. Five of the animals, including all of those showing protein pooling, showed little evidence of recent feeding, fluid main gut contents, and dilated digestive tubules.

Table 9. Summary of tissue changes in animals administered antibiotics, as total score of the change relative to the no of that tissue examined.

		Food trial 1 OTC only		Food combined 1				Bath 1				Food combined 2		
Tissue	**Change**	**Control (No examined)**	**OTC (in)**	**Control (in & on)**	**Amoxicillin (in & on)**	**OTC (in & on)**	**TMP-SDZ (in & on)**	**Control**	**Amox bath**	**OTC bath**	**TMP-SDZ bath**	**Control (in)**	**OXO (in & on)**	**TMP (in & on)**
foot	**epithelial loss**	1 (7)	9 (10)	7 (20)	6 (16)	5 (17)	14 (17)	9 (9)	7 (10)	8 (10)	5 (10)	7 (5)	12 (20)	8 (15)
	foot blisters		3	12	12	7	12 (esp on)	1	3	9	4	10	14	12
	focal greying,		1	3	4	5	13		2	2	3	2	5	5
	inflammation & other			vac 2	vac 2	aggreg 3	4	1	3	1, pr 1		2	3	
left kidney	**protein deposition**	6 (9)	3 (10)	14 (20)	3 (15)	5 (17)	10 (16)	4 (8)	8 (10)	4 (9)	9 (9)	3 (2)	29 (16)	22 (13)
	haemocyte pooling		1	16	2		12 (most in)	4 (1 tank)	2	5	1	1	23	16
	granular	1		2				1					5	1
	degeneration								1	2	1		1	3
right kidney	**pigment (melanin)**	8 (9)	10 (10)	26 (20)	24 (19)	29 (17)	29 (17)	18 (10)	21 (10)	15 (10)	10 (10)	11 (4)	37 (20)	20 (14)
	protein deposition			6	3	3	10	8	5	4	1		9	5
	degeneration							1 focal	1				1	
	thin tubules			1	2									
	dilution	1		2		5	4	2				1	1	
gill	**protein deposition**	0 (9)	0 (10)	6 (20)	2 (18)	2 (15)	8 (17)	6 (10)	2 (10)	8 (10)	7 (10)	0 (4)	9 (20)	10 (13)
	haemocyte pooling				1	2					1		2	
	degeneration	1											1	
	other			sl thick 3	sl thick 3	sl thick 4	sl thick 1		dil 2	1	dil 1		Sl thick 5	
heart	**protein deposition**	0 (9)	0 (10)	0 (20)	0 (19)	6 (16)	5 (17)	0 (10)	1 (10)	0 (9)	0 (10)	0 (4)	0 (20)	1 (13)
	haemocyte pooling			2			2 (esp in)						4	2
	degeneration							2	1	1			3	1
	granular / Ca deposit			Grey 2		Grey 2	3	1		1	1		1	2
	dilation, other		1	3		3	inflam 1	5	2	2	1	1	3	3
main gut	**fluid contents**	2 poor (9)	1 (10)	3 (20)	4 (19)	3 (17)	6 (17) Esp in	2 (10)	1 (10)	1 (10)	1 (10)	1 (4)	13 (20)	8 (13)
(filling)	**gas, dilation**	1 sl bloat?							3	1	1 sl bloat?			
digestive	**dilation**	0 (9)	1 (10)	3 (20)	0 (19)	6 (17)	5 (17)	4 (10)	6 (10)	4 (10)	4 (10)	0 (4)	9 (20)	6 (9)
tubules	**oedema**			1	2	2	1						3	2
	degeneration									3	1			
	granular								6	2			2	
perigut	**Increased density**	1 (9)	2 (10)	0 (20)	2 (19)	3 (17)	2 (17)	0 (10)	1 (10))	1 (10)	0 (10)	2 (5)	7 (20)	1 (13)
granulocyte	**Decreased density**			5			1	1	1			1	2	
bed	**Dilated spaces**			4	4	6	11	4	6	4	3	2	9	8
	degeneration								1					

All animals showed some diffuse dilation of the peri-gut haemocyte bed, especially in the animal showing most protein pooling. Overall one animal was described as showing wild oedema and protein deposition suggesting a major cardiovascular disturbance, and two others showing similar broad changes to a lesser degree.

All of the 7 animals receiving the potentiated sulphonamide mixture as a food coating showed some loss of epithelium. Mild foot blisters were present in 5 animals, 3 with grey precipitates. This was accompanied by mild inflammation in 2 animals. Left kidney changes were restricted to very slight protein in 3 animals, cell pooling in 2 (1 slight, 1 moderate). The latter showed no obvious protein pooling in the left kidney tissues, but showed protein strings in the dilated lumen. One animal showed heavy protein through the right kidney, without left kidney involvement. One right kidney showed dilated tubules, and pigment was variable. A little protein was present in 2 gills, these animals also showing pericardial dilation. Generally gut contents were better, with an overtly empty gut in only one animal. There were no changes noted in other organs except for variable peri-gut haemocyte aggregations in one animal, possibly associated with occasional bacteria seen near the gut tubules.

Oral absorption experiment 2:

Controls: The five controls all showed mild foot blister like dilations. One showed moderate left kidney protein and cell pooling, another slight protein only. Right kidney pigment was variable and one showed focal tubule dilation. No changes in gill or heart. Some food contents in all animals, though poor in one. Perigut haemocytes abundant.

Oxolinic Acid: Animals in both groups showed a similar level of mild foot blistering and grey precipitates to the controls. Of the 10 animals examined who received oxolinic acid incorporated into the food, 4 of the 6 left kidneys sectioned showed heavy protein deposition, and two showed minor protein deposits. The 4 markedly affected kidneys also showed heavy haemocyte pooling. Three also showed protein deposition in other tissues (right kidney, heart, oesophageal pouch and under foot epithelium; gill and right kidney in the other). The three severely affected animals were apparently not feeding, with fluid gut contents and some dilated digestive gland tubules, the mildly affected animal showing some areas of fluid filled gut. Peri-gut haemocytes areas varied from very abundant, to focally or uniformly dilated and depleted (2 animals).

Mild blisters were also present in 6 of the of the 10 examined from the OXO coated food group. Protein was present in all 9 left kidneys examined (heavy in 5, moderate in 2). Six also showed left kidney heavy haemocyte pooling (marked in 3 animals). Protein deposition was found in other tissues in 7 animals: atria (1); focally in gills (3) and in both gills (slight) and right kidney (heavy) in 4 animals. Haemocytes were also pooled in dilated areas of the affected gill. Bubbles were present in the gut contents of 2 animals, while another 6 showed a basically empty gut. The perigut beds were abundant overall with focal depletion and dilation, and more uniformly depleted in the other 2 animals.

Trimethoprim: Comprehensive histopathology was carried out on 7 abalone fed trimethoprim incorporated into the food, plus foot muscle from 3 others. Of the 10 foot muscles examined, 6 showed mild blistering, 2 with grey. All of the 7 left kidneys showed at least slight fibrillar protein pooling, with heavier pools in 3, these also showing haemocyte pooling, 2 showing some protein in gills. Haemocyte beds were generally abundant, occasional with semi-focal dilations, but there was focal haemocyte clumping or aggregation in 3 animals.

Foot muscle was examined from 10 animals receiving coated trimethoprim, with comprehensive examination of 9 of these. Nine of the 10 feet showed at least slight blister like tissue distension, 2 with slight grey precipitate. All except one of the left kidneys in section

showed at least slight protein, with more extensive protein pooling in this and other tissues (heart, gills and right kidney) in 6 animals. Five of these also showed haemocyte pooling in the left kidney. The gut was mostly fluid filled, as were many dilated digestive tubules. Haemocyte beds showed widespread to semifocal dilation in 6 animals, with clumping in two.

5.2.2 Histological effects after 2 hour bath treatment (bath experiment 1)

Controls: Under the conditions of culture system and bath, there were some changes from normal noted in the 10 controls. Fluid contents in parts of the gut of 3 animals suggested some animals had not been eating well prior or the 2-hour bath. Heavy pigment in the right kidneys in 4 0f 10 animals, and moderate pigment in another 2, plus some dilation of right kidney tubules (2) also suggested slight decrease in overall condition. A little protein deposition was present in the left kidney of 3 animals, including uniform though light protein deposition throughout one kidney. Slight to moderate protein was also present in 4 gills. Digestive gland tubules showed an increase in intra-tubule (4) and extra-tubular spaces (2). Heart was slightly dilated in half the animals. All these changes represent non-specific recent changes (probably reflecting recent handling and bath treatment) and a mild non-specific decrease in condition. A little blister like damage to one foot surface, probably reflects sampling damage.

Amoxicillin: Similarly, 4 of the 10 animals from the amoxicillin bathed group had not been eating well, showing poor gut filling and some dilated digestive gland tubules, which is regarded as a pre-bath condition. Right kidney showed heavy pigment in 7 of 10 animals. with some increase noted occasionally in peri-tubule spaces (this not recorded in detail in all animals). Protein noted in four left kidney, 2 moderately heavy, plus haemocyte aggregates in 2. Two gills were slightly dilated, two with protein precipitation. Foot showed similar levels of loss of surface epithelium (slight in 4 animals), and or blister like sub-epithelial tissue dilation (3).

What appeared to differ from controls was an apparent calcium containing precipitate, or slight overall bluing, in 4 animals. This type of precipitate or colour change was also noted in 2 hearts and one foot surface.

Oxytetracycline: Similar slight epithelial loss and mild foot blisters noted in 4 of the 10 oxytetracycline bathed animals, plus more marked blistering and tissue dilation in two animals. also showing tissue bluing of foot and elsewhere. Bluing also seen in several left kidneys, some with cell accumulation, one with marked protein depositions. Granular (calcium like) depositions were also seen in one heart. Overall it was the suggestion of basic precipitates (with the appearance often seen with calcium deposits in tissues), which appeared to differ from the controls.

Sulphadiazine-Trimethoprim combination: While there was some foot damage and dilated foot spaces in most of the 10 animals bathed with the potentiated sulphonamide, these did not appear to differ from the level seen in the controls except in one animal with marked foot damage and protein deposits in other organs. Bluing of foot tissues was occasionally seen, but overall to a less marked degree than in other organs.

There was a definite increase in protein in left kidney (5), this being severe in 4. This was sometimes accompanied by an increase in leucocyte pooling, and blueing of the tissues. Gills varied from normal to multiple small dilations, some with minor protein pooling / precipitation. The animal with severe foot damage showed very marked protein deposition in one gill, as well as the left kidney. Dilation and cell sparsity of the perigut region was common. Several showed gas bubbles (small) in the gut, though otherwise the level of gut activity was similar to controls.

5.2.3 Discussion

Confidence in the safety of the treatment is also a requirement for use and registration. Assessment of antibiotic safety is also essential. Registered antibiotics have undergone toxicity trials at normal dose for the animals covered by registration, so significant evidence of toxicity was not expected, especially at levels chosen to be at or near normal clinical activity. However there was a need to assess this as the levels used were generally the highest recommended for other marine purposed, which were sometimes high in relation to the levels used in freshwater, and for bath treatment in particular had the potential to alter water quality factors, affecting the respiratory environment. The study was also limited by the lack of firm criteria for assessing and interpreting tissue changes due to non-infectious agents in abalone: these studies contributed to developing such criteria.

Overall the level of tissue change shown by control animals was greater than expected, confirming the limitations of the holding and handling system. Such changes are in line with previous descriptions of environment related tissue changes (Harris et al., 1998b) and other observations on acute handling damage (unpublished).

Apart from the confounding factors of infection in several animals in the second experiment, there was little overall tissue change after 5 days of oral OTC administration, but the peri-vascular beds did show either a slight increase in density or variable dilation and depletion of these areas. Oxytetracycline was shown to have an effect on food consumption and a bi-phasic circulating haemocyte counts (see Section 5.4). The slight changes in the peri-gut haemocyte beds, apparently the main reservoir for haemocytes and granulocytes, probably reflect the changed stimuli for mobilisation of haemocytes and a slight haematopoietic response. It is noteworthy that OTC has an immunosupressive effect on fish (reviewed Treves-Brown, 2000, p76). Though the effects best studied are mediated through suppression of the adaptive immune response, it is reported also to have an inhibitory effect on mitosis and to cause a delay on monocytes responses. The animals receiving the coated food showed similar changes, though the mild vascular changes were slightly more pronounced. One change not seen in the controls was a slight bluing or grey discolouration of tissues, with fine precipitates suggestive of calcium deposits. Such deposits have been seen previously in degenerating tissues of abalone, and are regarded as likely to represent calcium precipitation. Such precipitates occur under some circumstances in dying cells of higher animals, but may be more common and occur at an earlier stage in abalone as tissue levels are high, and divalent ions are usually controlled.

Similarly, taking the occasional focal bacterial infection into account, there were only slight differences in the overall range of changes after amoxicillin absorption, though slight protein pooling was again more common in the coated-feed group.

Changes were more severe overall after the potentiated sulphonamide food, particularly with regard to the amount of protein and cell pooling in the left kidney. Dilation of the right kidney tubules in some animals implies increased urine flow, presumably to excrete the drug, and is a similar change to previous observations of right kidney tubule dilation with poor water quality (Harris *et al*., 1998b; A. Mouton, pers comm., 2003). In contrast, the left kidney changes of protein and cell pooling suggest tissue damage and / or possibly a slowed haemocyte flow. That this change is not seen to the same degree in the animals fed trimethoprim alone, at a much higher dose rate, indicates that this damage is predominantly related to the sulphonamide component. Sulphonamides are recognised as being excreted via the kidneys in other animals, and indeed have been used for renal infections because of this pattern of excretion, though concentration in this organ may lead to renal damage and sometimes crystal formation in (reviewed for fish, Treves-Brown, 2000, p 95 and 98). These findings, repeated to a lesser extent in the coated-food group and in the bath group, especially as severe protein

pooling / deposition, suggests this is a real toxic change which would require further assessment if sulphonamides were to pursued further.

Similar changes of left kidney cell pooling and left kidney or widespread protein deposition were noted in the oxolinic acid group, which were also feeding poorly, another indicator of an adverse reaction, indicating that this antibiotic, even if available, may not have been desirable.

5.3 Measurement of antibiotic and innate inhibitory activity *in vitro*

5.3.1 Development of a quantitative assay for innate antibacterial factors

5.3.1.1 Initial screen for suitable indicator bacteria

Factors present in naive animals appeared not to inhibit the growth of pathogenic strains of *Vibrio harveyi.*

An initial screen of 27 *Vibrio* type isolates from the system for bacteria sensitive to abalone haemolymph revealed that of these, only two isolates termed FF1 and Q1 appeared suitable. Only these showed inhibition zones of 2mm after 48 hours incubation on lawn plates of Vibrio nutrient Agar (VNA) medium with sensitivity paper discs impregnated with haemolymph.

Definitive speciation of these two *Vibrio* isolates was not possible. Q1 could not be typed. The closest match to FF1 was a *Vibrio viscosus*, but with only an 89% match (98% match being required for a definitive result). No *V. harveyi* were isolated from the system.

These two isolates were used subsequent in the initial tests, although their use was discontinued later as their sensitivity in the dilution assay was considerably less than that of *V. harveyi* to haemolymph used at that time.

Results from initial set-up trials gave a maximum haemolymph dilution for detectable inhibition with 24 hours incubation of 1:8. Most abalone showed inhibition only to 1:2 dilution. A dilution range of 9:1 to 1:8 was therefore selected for the initial antibiotic absorption trials.

5.3.1.2 Quantitative *in vitro* assessment trial 1.

Results from this trial were confounded by agglutination and bacteria other than the added test bacterial suspension. At best the haemolymph was seen to reduce rather than prevent overall bacterial growth. Results are summarised in Table 10.

Table 10. Results of test from *in vitro* Trial 1.

		Abalone 1			Abalone 2		
		FF1			Q1		
No	Dilution	1	2	3	4	5	6
A	1:1.1	A1	A1	A1	A1	A1	A1
B	1:2	G1	G1	G1	G1	G1	G1
C	1:4	G2	G2	G2	G1	G1	G1
D	1:8	G2	G2	G2	G1	G1	G1
E	-ve control	G2	G2	G2	G2	G2	C
F	+ve control	G1*	G1*	G1*	G1	G1	G1
G	Haemolymph control	A1	A1	A1	C	C	C

(G1-3 = Growth, 1=light, 2=medium, 3=heavy; C= clear; A1-3 = Agglutination, 1=light, 2=medium, 3=heavy).

Agglutination was present both in haemolymph control and in the lowest haemolymph dilution of the challenge test with one abalone / bacterial series, and with the lowest test dilution but not the control haemolymph with the other, suggesting this is a characteristic of haemolymph. Thus agglutination was present in most tubes with virtually undiluted haemolymph (including controls), but was not present with haemolymph dilutions of 50% or greater. Haemolymph from abalone 2 agglutinated only with the addition of bacteria, but occurred in abalone 1 whether or not bacteria were added. Heavy growth in the negative control tubes for this abalone indicate bacteria were present in the haemolymph and thus these tubes would also contain bacteria. The confirmatory step of inoculation and culture of microtitre well contents on VNA plates confirmed the presence of bacteria in all agglutinated wells.

Growth on VNA was absent from all but one of the tubes scored as clear, though a light growth was found from one haemolymph control from abalone 2. This plus the growth present in some of the negative controls for abalone 2 suggest this haemolymph may also have contained low bacterial levels.

The incidence of multiple types of bacteria from two wells indicated contamination from within the preparation process, as only single colony types were observed for the same abalone used in other wells, but the low number of such tubes did not suggest this was a major contributing factor to the spurious bacterial findings. Rather, these results indicate that bacteria were present in at least one haemolymph sample.

Little evidence of innate antibacterial activity was found, although results were difficult to assess as there was some suppression of the level of bacterial growth rather than complete cessation, which could indicate innate activity was present but overwhelmed by the number of bacteria used. Now was it possible to determine whether this apparent low growth at high haemolymph concentrations was due to suppression by innate bacterial factors or competition between the test and haemolymph carried bacteria.

5.3.1.3 Quantitative *in vitro* assessment trial 2

This trial was run with haemolymph of two other abalone from the same group titrated against both indicator bacteria to differentiate any differences due to the indicator bacteria or external contamination. Results are summarised in Table 11.

Table 11. Results of test from 20-12-00.

		1	2	3	4	5	6	7	8	9	10	11	12
		Isolate FF1						Isolate Q1					
	Dilution	Abalone 1			Abalone 2			Abalone 1			Abalone 2		
A	1:1.1	G1	G1	G1	G1	G1	G1	G1	G1	G1	G1	G1	G1
B	1:2	G2	G2	G2	G2	G2	G2	G2	G2	G2	G2	G2	G2
C	1:4	G2	G2	G2	G2	G2	G2	G2	G2	G2	G2	G2	G2
D	1:8	G2	G2	G2	G2	G2	G2	G2	G2	G2	G2	G2	G2
E	-ve control	G2	G2	G2	C	C	C	G1	G1	G1	C	C	C
F	+ve control	G1*	G1*	G1*	G1*	G1	G1*	G1	G1	G1	G1	G1	G1
G	Haemolymph control	C	C		C	C	C				C	C	C

(X = multiple bacterial types, G1-3 = Growth, 1=light, 2=medium, 3=heavy; C= clear; A1-3 = Agglutination, 1=light, 2=medium, 3=heavy, * = doughnut shaped growth; clear in centre).

Potential for antibiotic use in abalone

These results clearly indicate significant levels of bacteria in haemolymph from abalone 1 but not abalone 2, which multiplied in the presence of MBA. Isolate FF1 showed a non-uniform growth pattern under these conditions where no haemolymph was present.

Some reduction in growth was found with high levels of haemolymph, but whether growth is being promoted by high levels of MBA at higher dilutions, or suppressed by haemolymph is unclear from these results. Regardless of this, the results indicate very low levels of innate antibacterial activity against these bacteria in these two abalone.

These findings suggested modifications would be needed for an effective test system.

5.3.1.4 Experiment 3: Timed kill of bacteria by normal abalone haemolymph

This trial was undertaken to refine appropriate conditions for the test, to ensure the test system reflected the normal biological operating range for innate activity. The initial trial showed considerable variation between plate counts from replicate tubes (Table 12). This was repeated using 3 plate replicates / tube to evaluate whether this variation was due to pipetting error or bacterial clumping (Table 13)

Table 12. Results for timed culture of bacteria and abalone haemolymph.

No.		1	2	3	4	5	6	7	8	9	10	11	12
		Isolate FF1											
		Abalone 1						Abalone 2					
Row	**Dilution**	Time 0			Time t (4 h)			Time 0			Time t (4h)		
A	10:1 HL to bacteria	*	141 #	84	254	*	129	53	40	37	*	145	155
B	+ve control	+	+	+	+	+	+	+	+	+	+	+	+
C	-ve control	-	-	-	-	-	-	+	+	+	+	+	+

(*=blurred, indistinct growth (too many cultures to count). # 2 types of colonies visible on culture plate from this well)

Table 13. Results for timed culture of bacteria and abalone haemolymph using triplicate plates for each well to assess variability due to mechanical factors such as accuracy of pipetting and mixing.

Isolate FF1													
		Abalone 3						Abalone 4					
		Time 0			Time t			Time 0			Time t		
No.		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	10:1	2	3	6	54	*	34	8	109	18	*	49	47
	HL :	-	125	14	44	*	61	15	*	12	59	49	*
	bact	3	3	20	160	*	*	15	25	*	43	40	*
B	+veC	+	+	+	+	+	+	+	+	+	+	+	+
C	-ve C	+	+	+	+	+	+	+	-	-	-	-	-

(*=Blurred, indistinct growth).

Potential for antibiotic use in abalone

Bacteria were present in the haemolymph from abalone 2 and 3. The consistency of these findings, and the lack of overt mixed infection, suggests this is due to bacterial carriage rather than simple contamination at collection.

In all samples, either with or without bacterial carriage / contamination, there was either a static result or an increase after 4 hours incubation, though the increase was generally within the same order of magnitude, consistent with about one multiplication cycle in this period. The variability was too great for a definitive survival index, but indicative values from columns 8-12 of the second trial based on wells A8 and A9 demonstrated a 4 hour survival index of 68.7% and 87% respectively (mean 77.9%.). This indicates a lack of bactericidal activity within this period, but due to the short time frame, gives little indication of the bacteriostatic activity of haemolymph.

5.3.1.5 Experiment 4: The effect of filtration and incubation temperature

Results of visual assessment of wells for growth after of 48 hour incubation at 25°C and 15°C respectively are summarised in Tables 14 and 15. Subsequent plate culture results for filtered samples are given in Table 16

Table14. Visual growth assessment after 25°C incubation for 48 hours. (21-3-01)

No.		1	2	3	4	5	6	7	8	9	10	11	12
		Filtered haemolymph						Un-filtered haemolymph					
	Dilution	Abalone 1			Abalone 2			Abalone 3			Abalone 4		
A	1:1.1	-	-	-	-	-	-	+	+	+	+	+	+
B	1:2	+	+	+	+	+	+	+	+	++	++	++	++
C	1:4	-	-	-	-	-	-	+++	+++	+++	+++	+++	+++
D	1:8	-	-	-	-	-	-	+	+	+	-	-	-
E	1:16	-	-	-	-	-	-	-	-	-	-	-	-
F	1:32	-	-	-	-	-	-	-	-	-	-	-	-
G	+ve control	+	+	+	+	+	+	+	+	+	+	+	+
H	-ve control	-	-	-	-	-	-	++	+++	+++	+++	+++	+++

(+ = positive growth, - = negative growth, x = multiple colony types).

Table 15. Visual growth assessment after 15°C incubation for 48 hours. (21-3-01)

No.		1	2	3	4	5	6	7	8	9	10	11	12
		Filtered haemolymph						Un-filtered haemolymph					
	Dilution	Abalone 1			Abalone 2			Abalone 3			Abalone 4		
A	1:1.1	-	-	-	-	-	-	+	+	+	+	+	+
B	1:2	-	-	-	-	-	-	++	++	++	++	++	++
C	1:4	-	-	-	-	-	-	+++	+++	+++	+++	+++	+++
D	1:8	-	-	-	-	-	-	+++	+++	+++	+++	+++	+++
E	1:16	-	-	-	-	-	-	++	++	+++	++	+++	+++
F	1:32	-	-	-	-	-	-	+++	+++	+++	+++	+++	+++
G	+ve control	+	+	+	+	+	+	-	-	-	-	-	-
H	-ve control	-	-	-	-	-	-	++	++	++	++	++	++

(+ = positive growth, - = negative growth, x = multiple colony types).

Table 16. Plate growth analysis for wells with filtered haemolymph (21-3-01 test).

	15°C						25°C					
No.	1	2	3	4	5	6	1	2	3	4	5	6
A	+	+	+	+	+	+	+	+	+	+	+	+
B	+	+	+	+	+	+	+	+	+	+	+	+
C	+	+	+	+	+	+	+	+	+	+	+	+
D	+	+	+	+	+	+	+	+	+	+	+	+
E	+	+	+	+	+	+	+	+	+	+	+	+
F	+	+	+	+	+	+	+	+	+	+	+	+
G	+	+	+	+	+	+	+	+	+	+	+	+
H	+	+	-	-	-	+	-	-	-	-	-	-

(+ = positive growth, - = negative growth, x = multiple colony types).

Plate results indicate viable bacteria were still present in test cells without visible growth, but growth had been suppressed, resuming when removed from the test system onto nutrient agar.

The comparison of filtered and unfiltered haemolymph, especially from the negative controls, indicates that filtration removed bacteria already present in the haemolymph, but the growth pattern was not straight forward, especially at 25°C. Both sets of results suggest the test indicator did not grow as well in this system as contaminants / bacteria already present in haemolymph. (An alternative but less likely explanation is that the presence of the haemocytes and / or other bacteria interfered with the antibacterial activity or even actively promoted growth at this temperature. As these became more diluted, the innate bacteriostatic activity became evident.) The strong differences between filtered and unfiltered results suggest a greater level of innate activity against *Vibrio harveyi* isolate 2201 than against the haemolymph contaminants. Comparison with previous trials also indicates higher levels of activity against this pathogen than against the environmental isolates FF1 and Q1. The apparent differential suppression of the *V. harveyi* isolate up to dilations of at least 1:32 confirms this is a suitable indicator but that filtration of haemolymph to remove competing bacteria which may not be subject to the same level of suppression is essential for measurement of antibacterial activity against this species.

Results from the filtered samples suggest that there may be is an optimum concentration of haemolymph to support growth of the indicator bacteria. Thus aberrant results around dilutions of 1:2 at 25°C are discussed further below.

5.3.1.6 Experiment 5: Heat-challenged abalone

Results from the syringe bled unstressed control animals are summarised in Table 17. Levels of growth suppression varied between the 4 animals, abalone 1 showing clear suppression to at least 1:64, while other abalone showed suppression at 9:1 and generally at high dilution levels, but growth at lower levels.

Results from the foot-bled animals were confounded by agglutination in all negative (haemolymph) control samples. Growth suppression was restricted to 9:1 and occasionally 1:2 dilutions only. (See Appendix 3, for full results). However these results could also be influenced by the tissue factor involved in sample agglutination, giving false positive results. These results confirm that minimal tissue disruption is essential for this test, implying careful syringe bleeding, as well as prompt filtration to remove haemocytes.

Table 17. Results for unstressed (control) animals, bled by syringe (28-3-01). Haemolymph was filtered, and tray incubated at 15°C for 48 hours. Samples are in duplicate rows, ie. A & B are duplicates of abalone 1.

No.	1	2	3	4	5	6	7	8	9	10	11	12
	9:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	+ve	-ve
A	-	-	-	-	-	-	-	+	+	+	+	-
B	-	-	-	-	-	-	-	-	-	+	+	-
C	-	+	-	-	-	-	-	+	+	+	+	-
D	-	+	+	-	-	+	-	-	-	-	+	-
E	-	+	+	+	+	+	-	-	-	-	+	-
F	-	+	+	+	+	+	+	+	+	+	+	-
G	-	+	+	+	+	+	+	-	-	-	+	-
H	-	-	+	+	-	-	-	-	-	-	+	-

5.3.1.7 Experiment 6: Effect of heat inactivation on innate antibacterial activity.

Trays were examined at 24 hours, 48 hours and 96 hours.

At 24 hours, a light growth appeared to be present in positive controls and most tubes with haemolymph dilutions of 1:8 or 1:16 or greater, but the growth was too light for definate readings at this stage. Growth could not be detected on the 15°C plates.

At 48 hours there were few tubes showing negative growth (other than negative controls) in the 25°C plate, with a number of tubes including positive controls showing heavy growth. The 15°C plate (Table 18) showed better differentiation, with only light growth, though only low levels of inhibition compared to the previous trials. Both fresh and inactivated sera showed one abalone with inhibition up to a 1:2 dilution, with some inhibition up to 1:8 dilution. One heamolymph from each pair (fresh or heated) showed only sporadic inhibition of growth over this period.

Table 18. Comparison of growth inhibition by heat inactivated and fresh haemolymph. This tray was incubated at 15°C and read at 48 hours.

No.		**Ab no**	1	2	3	4	5	6	7	8	9	10	11	12
Dil			9:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	+ve	-ve
A	**Heat Inactivated**	**1**	1	1	1	1	1	1	1	1	1	1	1	-
B		**1**	1	-	1	1	1	1	1	1	1	1	1	-
C		**2**	-	-	-	-	1	-	1	1	1	1	1	-
D		**2**	-	-	-	1	1	1	1	1	1	1	1	-
E	**Fresh**	**3**	-	-	1	-	1	1	1	1	1	1	1	-
F		**3**	-	-	1	1	1	1	1	1	1	1	1	-
G		**4**	1	1	1	1	1	1	1	1	1	1	1	-
H		**4**	-	-	1	1	1	1	1	1	1	1	1	-

The 4 frozen samples, also incubated at 15°C showed incomplete inhibition to a similar level from one of the 4 animals, suggesting there was little effect of freezing on activity, though the conditions of this test did not provide a sensitive indicator for this effect.

5.3.2 Assessment of innate and antibiotic activity *in vitro*

5.3.2.1 Antibacterial activity during antibiotic food absorption trial 1. Oxytetracycline

Inhibition of *V. harveyi* occurred in all of the treatment animals at the 1:8 (the highest dilution used) while control treatments averaged inhibition at half this level, with some animals also showing inhibition at 1:8. The change in inhibition attributable to the antibiotic is therefore uncertain.

5.3.2.2 Antibacterial activity for antibiotic trial 2. Oxytetracycline, Amoxicillin and Trimethoprim/Sulphadiazine (oral and bath)

The presence of inhibition was tested at haemocyte dilutions ranging from 9:1 to 1:8, against one of the original local non-pathogenic indicator bacteria (*Vibrio* isolate FF1) and *Vibrio harveyi* isolate 2201, prior to the need for haemolymph filtration being established.

Inhibition against the local *Vibrio* isolate (FF1) was in general very low but did varied over the entire tested range, while not producing a pattern related to the treatments. Maximum inhibitory dilution varied in individual animals from 9:1 to 1 in 4, but this appeared only to reflect individual animal variation. This was also confounded by some negative control tubes also showed bacterial growth, especially those with apparently low inhibitory levels. This is consistent with the later findings on bacterial carriage and the need for filtration.

A similar but less marked effect was seen with the second indicator (*Vibrio harveyi* isolate 2201). All haemolymph including controls showed at least some inhibition, this reaching the test limit of 1 in 8 in many animals, including controls. While some tubes suggested a lower level of inhibition (maximum inhibitory dilutions of 1 in 2 or 1 in 4), negative controls for these tubes generally showed haemolymph contamination / bacterial carriage, again suggesting minimal inhibitory activity against the contaminants, but at least moderate activity overall against the indicator, *V. harveyi* isolate 2201.

There was insufficient haemolymph for re-testing at higher dilutions or with filtration. Neither maximum inhibitory levels nor the inhibitory contribution of the antibiotic treatments could be measured. Subsequent examinations of cohorts suggested innate activity was likely to have been high, well beyond the 1 in 8 dilution levels used.

Samples from the bath trial for these antibiotics were tested in a similar manner and produced similar results. There was minimal inhibition of the local *Vibrio* isolate (FF1), most samples showing inhibition only with undiluted haemolymph (9:1), with or without contamination. A few uncontaminated samples showed inhibition beyond this level but rarely to the test limit. In contrast *V. harveyi* isolate 2201 was usually inhibited at all test dilutions unless growth in the negative controls indicated contamination was present.

5.3.2.3 Innate antibacterial activity during antibiotic food trial 3. Oxolinic acid and Trimethoprim (food trial only)

A consistent pattern of inhibition at the 9:1 concentration occurred throughout this experiment. Only sporadic inhibition was noted for either controls or for medicated abalone. These results indicate not only that innate activity was low in these animals, but confirm the results from chemical analysis and *in vivo* treatment of natural infection, that the levels of oxolinic acid were very low detectable 24 hours after medicated food was withdrawn (which is presumed to mean very little was absorbed), and that trimethoprim, if absorbed at all from food, was inactive in abalone against this bacteria.

5.3.3 Discussion

Antimicrobial peptides have been defined as molecules of less than 10 kDa which show stoichiometric, as opposed to enzymatic, anti-microbial properties. A review by Borman (1995) concluded that they are important in the first line of the host defence system of many animal species, and that their value in innate immunity lies in their ability to function without either high specificity or memory.

Selection of indicator bacteria is critical to the outcome of a test designed to measure such *in vitro* antibacterial activity. There is recognition of at least several antibacterial factors in other invertebrates, including molluscs (for example, Charlet et al., 1996). They reported that it was probable that at least several antibacterial factors are present, targeting different bacterial surface components. On the expectation that at least some antibacterial factors are likely to target surface characteristics shared by most *Vibrio* species, it was considered that *Vibrio* species as an indicator would more closely predict the disease susceptibility of abalone to commercially significant diseases including *V. harveyi* infection. The experience of these trials clearly indicates a more selective activity towards *V. harveyi,* with little inhibition of other non-pathogenic *Vibrio* isolates. Negligible inhibition was found with most *Vibrio* isolates during the initial screen of 27 isolates, and inhibitory activity to the two most promising of these isolates was too poor to provide a sensitive and repeatable assay under these conditions.

It is significant that innate factors present in the first intake of naive animals (September, early spring) appeared not to inhibit the growth of the pathogenic strains of *Vibrio harveyi* tested at that time, though strong inhibition was present in haemolymph abalone brought into the system later in the summer. This information alone suggests that these factors are inducible, and the later intakes had been exposed to *V. harveyi* infection.

That the increased inhibitory activity in haemolymph from later intakes was evident against *V. harveyi* but not the two *Vibrio* strains initially use as indicators, confirms the selectivity of the response. These findings are consistent with more recent reports of a selective and adaptive response to pathogenic but not benign bacteria in molluscs, including abalone (K. Birkendorff, pers. comm.; Wang et al., 2003) and even sponges (Muller et al., 2004).

The question of specificity of such induced responses is still under question. How many separate antibacterial factors are present in abalone, the range of species that share the same target surface regions as *V. harveyi,* and whether these surface target molecules reflect or confer pathogenicity is still largely unknown. At the time of these trials the expectation was of a small number of innate factors targeting common surface receptors likely to be shared by many bacteria, but as more factors are found the likelihood of a more specific response increases. In retrospect the expectation of sufficient shared regions between pathogenic species and the non-pathogenic bacteria used initially for these to be useful indicators may have been unrealistic. Such assumptions have been common and underlie the broad-scale screening of mollusc haemolymph for antibacterial activity that could be used in humans, culminating in research proposed by Ruggeiri (1976) in a search for “Drugs From the Sea”.

As activity against bacteria isolated from the culture system was generally very low, it is likely that characteristics of abalone tissues resulting in an unsuitable internal environment for these bacteria may be more important than specific inhibitory substances in preventing the growth in abalone tissues. It follows that it is unlikely that such bacteria would reach a tissue concentration that induces these innate factors. It is noteworthy, though, that although these two strains (FF1 and Q1) did share some inhibition recognition sites with each other and with *V. harveyi*, the remaining 25 presumptive *Vibrio* isolated showed neither inhibition with haemolymph or pathogenic invasion.

Potential for antibiotic use in abalone

Development and use of a quantitative assay based on serial dilution was hampered by the inexact science of bacterial dilution, variable but apparently frequent sub-clinical bacterial carriage, and significant variation in the levels of innate antibacterial factors. Nevertheless this series of experiments provides considerable information on the nature of these factors.

Results from the first quantitative trial were difficult to interpret as there was some suppression of the level of bacterial growth rather than complete cessation, and agglutination was present in tubes with a high proportion of haemolymph. Confirmation of bacterial growth in all agglutinating tubes with culture onto VNA plates indicated both haemolymph and bacteria were required for agglutination to take place in these tubes. There was also an indication of both low levels of contamination during the test process and of probable bacterial carriage by one of the two abalone used for this test. It was not possible to differentiate between contamination during sampling and bacterial carriage by the clinically health abalone, though higher levels of multiple bacterial types would be expected if this was primarily due to contamination. The low but measurable levels of bacterial inhibition (mostly at maximum haemolymph diluents of 1in 2, occasionally to 1 in 8), suggests that normal background levels of innate factors may be quite low prior to induction after exposure to potentially pathogenic bacteria. Although this needs to be interpreted with caution, given that the total activity may represent a number of similar factors targeting different surface factors, this is in line with the initial indication that naive animals showed no activity against several *V. harveyi* strains in the initial screening process.

That growth even in the negative control samples was higher than the positive controls suggests that the bacteria already present in the haemolymph out-grew the indicator bacteria (especially Q1). This could indicate they were possibly already present at very high levels, and were at most minimally suppressed by the abalone haemolymph factors present. One could speculate from the level of agglutination seen, that the uneven result in the negative control for abalone 2 (one clear well) could be due to agglutination of bacteria and cells and / or proteins commencing prior to addition to the tubes, so that bacteria were present in clumps and therefore unevenly distributed. The overall results suggest that both abalone carried bacteria, and that both haemolymph from both abalone contained factors which agglutinated at high levels in the presence of bacteria. The results suggest this was precipitated by Q1 but not contaminating bacteria.

It is unclear if factors initiating agglutination of haemolymph of these two abalone are the same as those causing agglutination in haemolymph samples collected by foot muscle section from Experiment 5 of this series. The latter suggests that factors released from damaged abalone tissues are implicit in this agglutination process. Whether or not there was sufficient cell damage during haemolymph collection for Experiment 1 is not clear: the collector was experienced in bleeding abalone but only relatively small animals were available for these experiments. Agglutination following tissue damage is not unexpected and in line with functional cascades demonstrated in many animals, whereby products of tissue damage precipitate clotting mechanisms. Despite the established dogma that abalone haemolymph does not possess clotting factors, haemocyte clots in exposed haemolymph are readily observed, though the factors mediating this are not well studied. Haemolymph for Experiment 1 had not been filtered, so haemocytes may have been involved in the agglutination reaction. Regardless of the biological significance of this process, haemolymph for inhibition tests should be drawn from vessels or sinuses, rather than from transected foot muscle, to avoid the products of cell damage. Low and possibly variable amounts of tissue damage products may be expected with haemolymph collection via a syringe, but good technique will minimise this.

Similar results were obtained from the second quantitative trial, though no agglutination was seen. Results with both indicator bacteria suggest one of the abalone used for this trial carried

bacteria, the other did not. Low levels of haemolymph from this animal appeared possibly to enhance growth, compared to positive bacteria controls, though this was not evident at the highest haemolymph concentration. One possible explanation for the plate tests suggest bacteria carried by abalone 1 were of a type that was either similar to or suppressed by isolate FF1, but was dissimilar to and not suppressed by isolate Q1.

The two trials of Experiment 3 were undertaken to determine whether the antibacterial factors being assessed in this system were bacteriostatic (suppressing bacterial growth) or bactericidal. The short incubation period of 4 hours was expected to be sufficient if action was primarily bactericidal, but to have little overall effect if bacteriostatic. Although some of the plates in the did not show discrete colonies and there was some variation between replicates, the overall trend was for results after 4 hours to be a similar order of magnitude to the pre-incubation counts. Some increase was apparent after incubation with abalone haemolymph in trial 1, and bacterial carriage or contamination was demonstrated in abalone 2 but not abalone 1. Results were more variable but with no overall change in the second of these trials, with bacterial carriage or contamination in abalone 3 but not abalone 4. Overall the results suggest any suppression of bacterial growth of FF1 with the longer incubation times was likely to be due to bacteriostatic rather than bactericidal activity.

Because bacterial carriage or contamination had been shown to occur in approximately half the abalone, filtration was introduced and used for the remaining *in vitro* tests. Experiment 4 clearly indicates filtered haemolymph did inhibit bacterial growth at most of the dilutions tested.

The pattern of inhibition was more clearly apparent than for haemolymph where bacteria were present, though anomalies at low dilutions occurred in both groups. In the two unfiltered haemolymphs, the number of bacteria present after 48 hours exceeding the number of *V. harveyi* added as indicator. (Indeed *V. harveyi* did not appear to grow well at 15°C in this system, compared to haemolymph contaminants. However plate culture confirmed that viable bacteria were still present.) The pattern overall suggests suppression of both *V. harveyi* and bacteria already present at high haemolymph dilutions, with suppression more evident when *V. harveyi* alone was present.

The pattern at 21°C of negative growth with low concentrations of haemolymph, despite growth at higher concentrations, again suggests that haemolymph may promote growth at optimum concentrations, giving a diphasic response with dilution. Haemolymph is not essential to growth, as this was observed in positive controls with the same number of added *V. harveyi* in the absence of haemolymph. This effect was only noted at 21°C. This may be a combination of supply of nutrient / growth factors, and levels of innate inhibitory factors. Variability appears reduced by the longer incubation time, presumably as this assesses both bacteriostatic and slow bactericidal activity. In all animals, the suppression of *V. harveyi* by dilute haemolymph extended beyond the dilution range of 1:32.

Experiment 5 differed mainly in the use of haemolymph collected from severed foot muscle from some animals, rather than collected via a fine syringe, resulting in agglutination as discussed above. The method of haemolymph collection influence this test, with agglutination in the haemolymph controls in all three of the trays of haemolymph withdrawn from the foot and apparently low levels of bacterial growth inhibition from these samples, so it was not possible to draw conclusions about the effect of the heat treatment some of these animals had undergone (Handlinger et al., 2004). The few samples which were collected via syringe and needle from the cephalic sinus indicated inhibition of *V. harveyi* growth was variable but in some samples was still present at haemolymph dilutions of 1:512. The cause for the aberrant inhibition at the higher haemolymph dilution levels is due to innate activity in the haemolymph, or to other factors, for example in the MHB broth is unclear.

Interpretation of Experiment 6, designed to examine heat stability of the inhibitory factors, may have been compromised by a decision to increase the incubation time. Whether or not this resulted in late growth of bacteria following initial suppression, the level of inhibitory activity shown in either group after 96 hours incubation at 25°C was too low to assess whether these factors are heat labile. Some inhibitory activity was demonstrated after incubation at 15°C, but only as scattered activity throughout heat treated and untreated haemolymph wells. The results suggest that low activity was likely to be a reflection of the increased incubation time. Minimal activity was also found with the frozen samples incubated for 48 hours at 15°C, but any reduction in activity due to freezing was masked by the minimal activity shown under these conditions. For this reason neither the effect of heating, or of freezing, could be determined from these samples. Results after 24 hours showed slightly more inhibition in some animals, with no apparent reduction after heat treatment although a higher initial level of inhibition would be required to verify this.

The comparisons of inhibition due to antibiotics and innate factors were also in general disappointing. There was some apparent increase in antibiotic activity due to oxytetracycline was evident in the initial experiment, but the normal population was too variable for a clear cut measurement of this. For both the oral and bath treatment trials for oxytetracycline, amoxicillin and trimethoprim/sulphadiazine mixture, inhibition of the *Vibrio* isolate FF1 was too low and variable to be useful, while maximum inhibitory levels for the *V. harveyi* isolate were in general beyond the test dilutions used. That the lowest levels of inhibition against both indicators were generally in samples where the negative controls indicated bacterial carriage or contamination suggests inhibition levels against the contaminant to be lower than to either indicator. An alternate explanation is that the presence of bacterial carriage had removed some of the antibacterial compounds, thereby lowering the levels. Clearly the tests were run over too narrow a dilution range, and given the variation, probably with insufficient numbers of animals, to measure the level of antibiotic activity. These tests did, however, highlight the differential nature of the natural inhibition to different bacteria.

These constraints had less impact on the last of these trials, to measure antibiotic activity after oral administration of oxolinic and trimethoprim. The lack of innate inhibition in this test enhanced rather than obscured the lack of inhibition by the antibiotics. These results are consistent with the analytical results for absorption of oxolinic acid from food, and the lack of efficacy obtained by trimethoprim medication in feed of the natural *V. harveyi* outbreak (section 5.4.3).

5.4 Assessment of antibiotic efficacy *in vivo*.

5.4.1 Development of experimental infection model

5.4.1.1 Infection model trial 1:

Retrospective Miles & Misra counts indicated the actual dose rates of bacteria used in Trial 1 were 8.33×10^{1}, $^{3.83} \times 10^{2}$, 2.87×10^{3}, and 2.64×10^{4} (Table 19). No deaths occurred during the period of this trial. Bacterial culture and histological assessment (below) indicated that the bacteria were still present at 6 days. There were no significant differences in haemocyte counts.

Table 19. Abalone infection model experiment 1 (16°C).

Nominal count	Miles-Misra count	No. cells in 100 µl	Mortalities (from 6)	Haemocyte counts (20µl)	Mean Haemocytes / ml
10^5	2.64 x 10^5	2.64 x 10^4	0	81.0±4.0	4050
10^4	2.87 x 10^4	2.87 x 10^3	0	129.0±28.6	6450
10^3	3.83 x 10^3	3.83 x 10^2	0	82.0±30.5	4100
10^2	8.33 x 10^2	8.33 x 10^1	0	120.4±15.5	6000
0	0		1	94.3±18.1	4700

5.4.1.2 Infection model trial 2

In Trial 2, Miles & Misra counts did not provide useable results, but only two animals died after injection with a nominal dose of 10^6 bacteria or less (both at 10^5 bacteria). (Table 20) All but one animal above that level died (nominal dose 10^7). *V. harveyi* was recovered from abalone receiving non-lethal doses of the bacteria, indicating that they remained infected for at least the 6 day duration of the experiment. There were no significant differences in haemocyte counts within the experiment, though overall the counts at 21°C were higher than counts across the two experiments at 16°C.

Table 20. Abalone infection model experiment 2 (21°C). Miles/Misra counts did not provide useable results.

°C	Nominal Bacterial count	Miles-Misra count	No. cells in 100 µl	Mortalities (from 6)	Haemocyte Counts (20µl)	Mean Haemocytes / ml
21°C	10^9	-	10^8	6	-	
21°C	10^8	-	10^7	5	-	
21°C	10^7	-	10^6	0	180.5±21.6	9025
21°C	10^6	-	10^5	2	154.3±58.8	7697
21°C	10^5	-	10^4	0	158.2±16.9	7910
21°C	0	-	0	0	207.6±11.6	10,380
16°C	0	-	0	0	139.8±34.9	6990

5.4.1.3 Characterisation of *V. harveyi* artificial infection.

Histopathology of infection model trial 1

Histological assessment of these abalone indicated that subtle early changes could be attributed to the treatments becoming progressively more marked with bacterial dose, though only one overt bacterial focus was seen.

Four blank infected controls showed little variation from normal. Hearts and main gut were slightly dilated (seen also in animals from treatment groups). There was only occasional moderate pigmentation of right kidney cells (one animal). Only one left kidney showed a little light contracted protein, and one showed a moderately dense perigut haemocyte bed, indicating a moderate recent stimulus to haemocyte production. No foot surface blistering was seen.

Potential for antibiotic use in abalone

Of the 6 animals receiving (a notional) 10^2 *V. harveyi* bacteria 6 days prior to sampling, 3 showed slight dilation of surface foot spaces (blistering), this being accompanied by a slight diffuse increase in haemocytes in this region of the foot. Moderate to marked pigmentation was seen in right kidney cells of 3 abalone, perigut haemocyte beds were dense in 3 abalone, moderate in one, dense contracting protein was seen in one gill and in the left kidney of another which also showed a haemocyte clot at the base of the gill. No bacteria were detected.

Foot dilation or blistering was not evident in the 6 animals which received 10^3 *V. harveyi*, though one showed small areas of semi-diffuse haemocyte increase in the foot. A moderate to marked increase was seen in 5 of 6 right kidneys; haemocyte beds were moderate to dense in 3 animals and haemocytes appeared slightly aggregated in one of these. Small areas of protein deposition were seen in one atrial tip and one left kidney.

4 of the 6 animals receiving 10^4 *V. harveyi* showed a slight semi-diffuse haemocyte increase in the foot, but no overt blistering. Moderate to dense haemocyte beds noted in 5 of the 6 animals, those these did vary in character, most being mixed as expected, but one showing few granulocytes, another showing occasional pycnotic cells. No overt bacteria were seen though there was a suspicion of dead bacteria in areas of vacuolation and pigmentation in one ventricle. This abalone also showed increased haemocytes between right kidney tubules, with occasional pycnosis. Moderate pigment present in right kidney of 3 abalone, heavy in another. Protein deposition limited to slight globulated (old?) protein deposit in one left kidney.

The 6 animals receiving a notional 10^5 *V. harveyi* dose included 4 with small foot blisters and very slight foot dilations in the remaining 2. No foot abscesses were seen though one showed a small abscess like reaction round bacteria in the right kidney. Perigut haemocyte beds were abundant and active in all animals, though this was focally dilated in the abalone showing the small right kidney abscess.

Histopathology of infection model trial 2

Blank injected animals (3 examined) showed more non-specific changes than trial 1, but not specific indications of an infection. These changes include small blister like foot dilation in 2 animals, variable perigut beds, with some intense foci of haemocytes with a focus of apparent haemocyte aggregation in one animal and areas with a dilated appearance. Overall these areas appeared more active than in the previous experiment. Other changes in the blank injected animals included focal protein in the lamina propria of one oesophagus, more generalised though slight protein deposition in another, including right and left kidneys. The other animal showed a small cellular reaction on the auricle. All showed moderate right kidney pigment.

The four animals receiving a notional 10^5 *V. harveyi* that were examined histologically all showed foot dilations and blisters, but these were accompanied by epithelial loss in two, deeper foci of dilation including round ganglia in one, and haemocytes aggregation in vessels in another. Perigut areas were all variable. As well as dense areas dominated by small haemocytes, three showed areas of depletion and dilation, two showed foci of apparent haemocyte aggregation / reaction. Right kidney pigmentation was not marked, being moderate in one but slight or absent in the others. Of the two left kidneys examined, one showed haemocyte pooling and a grey fibrillar appearance (b ut no bacterial filtration), the other contracting protein. Protein was also present in one gill, in quantities judged to have obstructed blood flow in this side of the gill. Pericardial dilation was also present in this animal (possibly a consequence of partial gill obstruction).

One animal in this group also showed multiple small granuloma-like reactions in digestive gland tubules. The time frame of reactions with this appearance in abalone has not been established and a relationship to *V. harveyi* infection is uncertain.

Of the three animals examined after receiving a notional *V. harveyi* dose of $10^{6,}$ one showed small foot abscesses as well as many blisters with slight inflammation apparently approaching this stage, but the other two showed only slight surface loss and minor blistering. This animal also showed a focus of inflammation and necrosis in the heart (which has not previously been associated with *V. harveyi* type infection, but has been seen with other forms of vibriosis). Some basophilic (calcium like) deposit was associated with this (probably a result of localised ion release from ruptured cells). The left kidney of this animal showed haemocyte pooling plus flocculated protein and loss of surface nuclei or marked eosinophilia of surface cells (which represents another new observation). Mild degeneration and haemocyte increase was also present in the gills and the right kidney was dilated.

A second animal in this group also showed a change not previously associated with *V. harveyi* infection, as a focal necrosis of digestive gland tubules including disruption to the basement membrane and a surrounding haemocyte reaction. Focal reactions were also present in the auricle and right kidney, with moderate tubule pigment in remaining areas of this organ. Perigut haemocyte beds were variable, with focal haemocyte aggregation.

The other animal in this group showed less severe change, similar to those described above with diffuse reactions in the perigut haemocyte beds and moderate right kidney pigment.

The four animals that received a notional 10^7 *V. harveyi* dose showed only pallor, epithelial loss and some blistering of the foot. One showed gill necrosis and a massive reaction on one side of gill, with apparent fusion across adjacent lamellae with pycnotic cells in this area. Another showed heavy protein deposition and haemocyte pooling suggested the protein deposits had impeded flow. All showed active perigut haemocyte beds with increased small haemocytes, with variable levels of depletion and tissue dilation, and one adjacent area of digestive tubule disruption and reaction.

All of the high-dose animals died, and few were suitable for histopathology, precluding a systematic examination of these groups though three were examined. These showed foot blisters rather than abscesses, a similar range of haemocyte reactions, variable protein deposition in gills and kidneys. Two showed multiple digestive gland granulomas.

5.4.2 Antibiotic treatment of artificially infected abalone

5.4.2.1 Effect of treatment on survival

Mortalities began appearing on day 2. All animals receiving bacterial injections but not treated with OTC and all of one tank of animals receiving bacterial injections but treated with OTC, plus 7 of the 10 animals in the other treated tank died between days 2-4. The three remaining abalone in this tank survived the trial period. There were 4 deaths overall in the controls, with one abalone dying in the seawater control injected treatment group on day 2, 2 more deaths in this tank on day 6 and one death in an untreated abalone held at 21°C on day 6. Tank details and results are summarised in Table 21 and Figure 11.

Table 21. Treatments and survival, OTC treatment of artificial *V. harveyi* infection.

No	Treatment	No / treatment	Survival (%)
1	OTC in diet, bacteria injected	20	15
2	OTC in diet	10	100
3	Normal diet, bacteria injected	19	0
4	Normal diet, blank injection	18	83
5	No treatment, 21°C	15	94
6	No treatment, 17°C	9	100

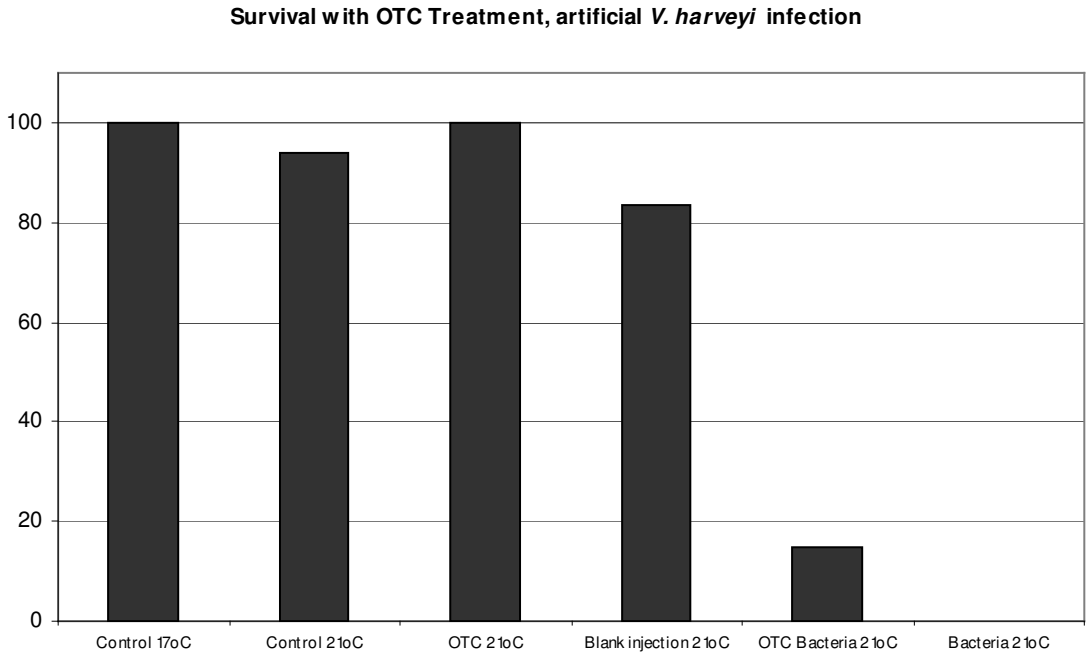

Figure 11. Effect of antibiotic treatment on survival of artificial *V. harveyi* infection.

In this experiment food consumption was not suppressed by OTC treatment or by the stress of injection, with average consumption in all group of 0.8-1% body weight/day. In fact injection, which presumably constitutes a stress, appears to have slightly stimulated appetite. Similarly, injection seems to have also increased haemocyte counts. Mean haemocytes / ml of untreated animals at 21^{o}C was 5500 $\pm$ 1095 (n=10); of blank injected animals 9087 $\pm$1463 (n=4). Average for untreated animals at 17^{o}C was 6804 $\pm$2801 (n=11). Haemocyte count from the only surviving OTC treated and bacterial injected animal from which sufficient haemolymph could be obtained was 7800.

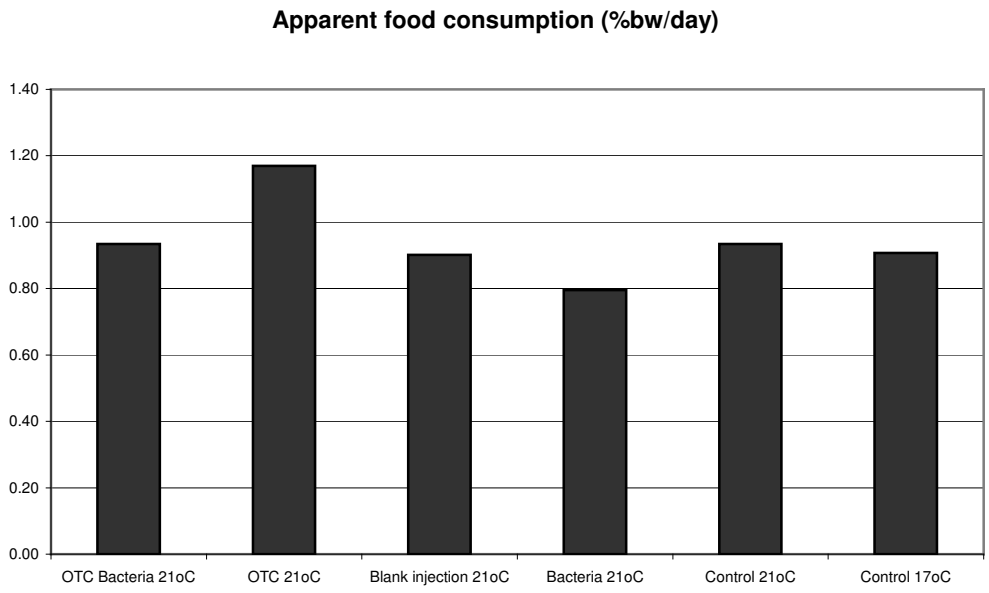

Figure 12. Effect of intravascular injection, *V. harveyi* bacteria and oxytetracycline treatment on food consumption.

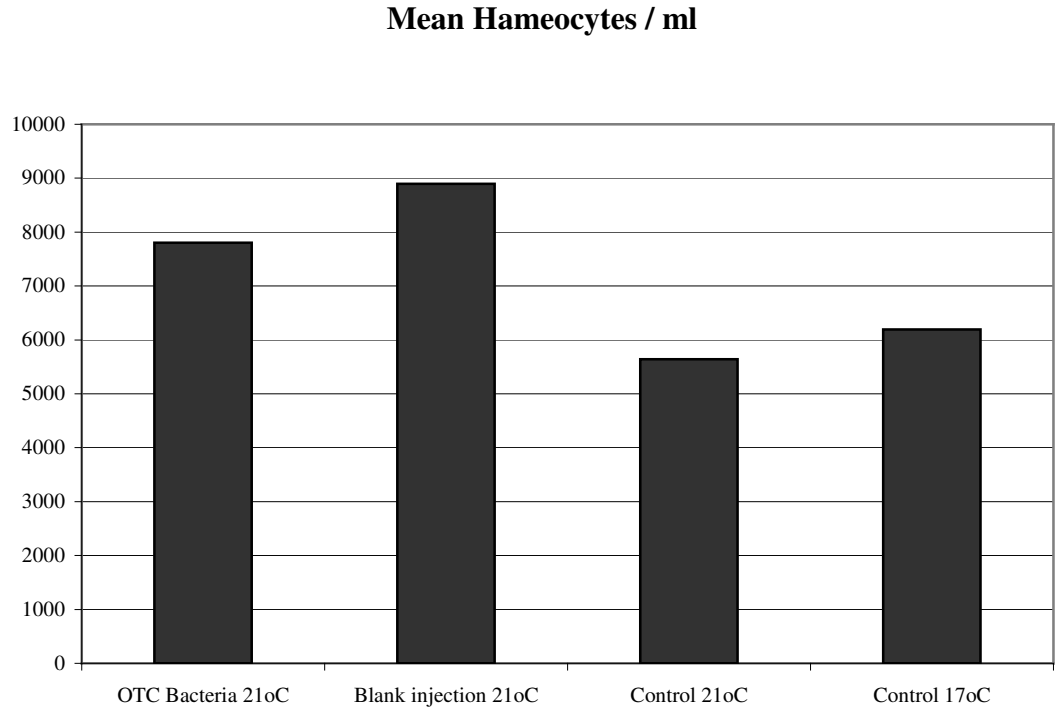

Figure 13. Effect of injection, temperature and antibiotic treated artificial infection on circulating haemocyte count. (Note OTC + bacteria treatment was from 1 animal).

5.4.2.2 Antibiotic residues from artificial infection trial.

Pooled samples of five abalone from each of the two tanks receiving OTC but no bacteria, which had been collected shortly after the bacterial injections, showed whole body OTC residues of 4.7 and 4.0 mg/kg.

5.4.2.3 Innate and antibiotic related *in vitro* inhibition from artificial challenge trial

It was not possible to obtain haemolymph samples from dead abalone, so there were no samples collected from artificially infected but untreated abalone (Treatment 3 as shown in Table 22), all of which died. Samples were available from only one tank of the OTC treated infected group. The majority of samples from all untreated groups (23 of 28 tested) showed inhibition only at 9:1 haemolymph to bacterial suspension, and none showed inhibition at higher concentrations (Table 22). Five of the 7 abalone tested after OTC without infection, (Treatment 2 of Table 2) showed higher levels of inhibition, with maximum inhibition dilutions ranging from 9:1 (2), 1:2 (1); 1:4 (1); 1:32 (1) and 1:264 (2). Only one of the 3 infected and OTC treated animals tested showed inhibition, and this only at the 9:1 haemolymph to bacterial suspension ration.

Table 22: *In vitro* bacterial inhibition of uninfected normal and OTC treated animals from artificial challenge trial.

Temp	Diet	Inject.	No tested / tank	9:1	1:2	1:4	1:8	1:16	1:32	1:64	1:132	1: 264	1:528
21	Normal	None	6	6	0								
21	Normal	None	5	3	0								
21	Normal	Blank	5	4	0								
21	Normal	Blank	5	5	0								
21	OTC	None	4	4	3	3	3	3	3	2	2	2	0
21	OTC	None	3	3	2	1	0						
21	OTC	Bacteria	3	1									
17	Normal	None	2	1	0								
17	Normal	None	5	4	0								

5.4.2.4 Histopathology of OTC treated artificial *V. harveyi* infection

Controls, 17°C Background changes in the foot of the four untreated animals at 17°C examined histologically, included marked foot dilations (blisters) in one animal, without bacteria or inflammation, but only occasional focal pallor in other animals. Two showed areas of dilation and distension of the perigut haemocyte bed, with aggregation of some of these in the animal showing blisters. One showed a dense protein deposit in one gill (probably related to handling damage). One showed occasional digestive tubule granulomas, which is presumed to be an unrelated pre-existing condition. The other changes are common minor changes which may reflect handling damage and minor stresses.

Controls, 21°C: Ten 21°C untreated controls were examined, including the one death on day 6. This animal showed dilated foot spaces but no obvious bacteria. There was extensive slough into the gut tubules and ducts, the cause of which was not obvious. The live animals (day 7) were similar to the 17°C controls, though slight protein deposition was seen in gill vessels of four animals, plus occasional small foci in other tissues in one of these.

Blank injected controls: Seven of the blank-injected controls were examined, including four which had died. Of these, the animal which died on day 2 showed a major disruption of one gill, including protein deposition, cell pooling in the vessel behind this and necrosis (which may be secondary to inhibited haemolymph flow). There was a light scattering of bacteria associated with autolysis of the gut, but no indications of primary bacterial involvement. The animal had intra-epithelial granulomas, apparently pre-existing and non-significant. Overall this death appears likely to be the result of mechanical damage or similar during injection. In contrast the blank injected animals which died on day 6 both showed abscesses (early in one, well developed in the other). Contamination during injection appears likely.

There were no indications of infection in the remaining blank injected animals. One showed minor focal erosions and reactions in one gill, another slight protein deposition in the perigut region (both possibly reflecting mechanical injection damage).

Artificially infected, untreated: Of the animals which received bacteria but no antibiotic, all of 5 animals dying at day 2 which examined histologically showed bacterial foci, mainly through the foot, some clearly adjacent to haemolymph vessels, and occasionally in gill. Reaction was absent or occasionally slight. Other findings included deposits suggestive of calcium ions in heart and left kidney, occasionally gill.

Animals dying at day 3 were similar. The six animals examined showed in general fewer bacteria through the foot with slight reactions in foot and occasionally gills and heart, though one showed multiple foot abscesses. One autolysed animal was suggestive of bacterial localisation in or round gut with subsequent gut degeneration and reaction.

By day 4, both animals examined showed abscesses, though one of these showed only one small abscess with most bacteria in the perigut haemocyte bed. The single animal examined at day 5 showed abscesses plus heart reactions.

Bacteria plus OTC: Animals dying after artificial infection, despite OTC treatment were similar. Two animals examined from day 2 and two of those from day 3 showed foot bacterial foci, occasionally bacteria in the ventricle, and some calcium like deposits and dilations of the perigut haemocyte bed. A third animal dying at day 3 did show foot abscesses as well as bacteria in the ventricles, apparent calcium deposition and dilated haemocyte beds. The single animal examined on day 4 also showed abscesses, as well as apparently more recent secondary bacterial foci in the foot, with minimal reaction.

No abscesses were seen in the one treated survivor that was examined from the 7 day sampling.

5.4.3 Antibiotic treatment of natural infection

5.4.3.1 Effect of oxytetracycline and trimethoprim treatment on survival

Total pre-treatment cumulative mortality had reached 56% (though mortality rate was decreasing), by the time treatment commenced on Day 18 post transfer. (Figure 14).

Mortalities continued in all three treatment groups into the treatment period, but ceased in all groups before the end of the trial (Figure 15). Within the first 7 days of treatment there had been a further 35% mortality in the untreated animals (23 of 66 animals), 25% deaths (15/60) in the trimethoprim treated group of animals, and 12% (7 / 60) in the OTC treated animals. Between treatment days 7 and 10, there were a further 2 mortalities in the controls, 1 from the OTC treatment, and 6 from the trimethoprim treatment, bringing the total mortality during treatment to 38% for controls, 15% for OTC and 38% for trimethoprim. In the post-treatment period (day 10 - 26), there were only two further mortalities in the residual animals remaining

after the day 10 sampling (1 at day 13 from 31 remaining controls, and 1 at day 11 from 29 trimethoprim treated animals), with no deaths from the 39 remaining OTC treated animals.

The significantly different treatments were identified using Fishers LSD post-hoc test. Arcsine –square root transformed data showed mortality from the oxytetracycline treated group to be significantly different from the controls and trimethoprim treated groups. (p=0.053)

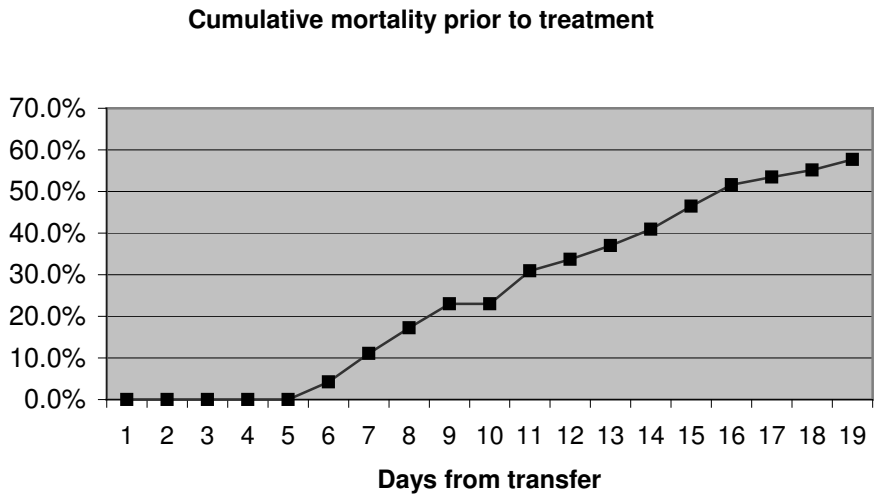

Figure 14. Cumulative mortality pattern due to natural *V. harveyi* infection, prior to commencement of antibiotic treatment.

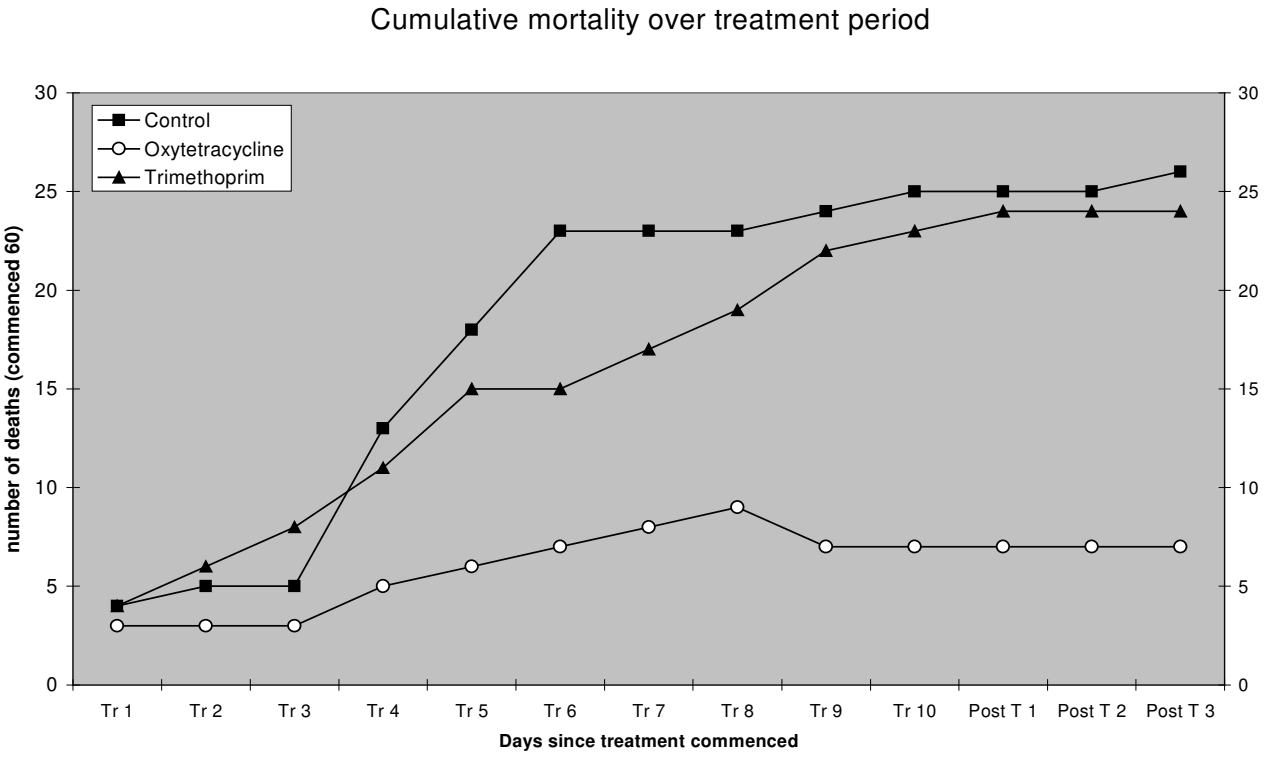

Figure 15. Cumulative mortality in antibiotic treated and untreated naturally infected abalone.

5.4.3.2 Effect of oxytetracycline and trimethoprim treatment on bacterial carriage

Identification of bacteria from these and other freshly dead or moribund animals during the course of treatment confirmed *V. harveyi* was among the multiple species present (some of which could not be speciated). Bacterial cultures on animals removed at the end of the treatment period (day 10-11), confirmed *Vibrio* species of bacteria in haemolymph samples from 8 of 9 adequate control abalone, 10 of 12 trimethoprim treated abalone, and 9 of 12 OTC treated abalone. The majority of the cultures selected for identification were sucrose positive. The strain of V. harveyi that causes blister disease in abalone is sucrose negative.

Five recently dead animals were examined histologically and were typical of *V harveyi* type infection (Handlinger et al., 2005). All showed typical foot abscess formation, to varying degrees. Bacteria were also present in all 3 left kidneys in section, ranging from occasional to marked in association with heavy protein deposition and in one heart. Their perigut haemocyte beds showed areas of depletion and dilated spaces.

In samples taken 18 days post treatment, sucrose positive *Vibrio* species were cultured from the haemolymph of 11 of 12 untreated control abalone, 7 of 12 trimethoprim treated abalone, and 9 of 12 OTC treated abalone. *V. harveyi* was not therefore the main isolate and may not have been present.

5.4.3.3 Tissue levels of oxytetracycline during treatment

OTC was undetectable in the controls (level of sensitivity 0/05 mg / kg). OTC was detected at low levels (0.79 mg/kg) in pooled gut tissue of abalone dying on Day 1 of treatment (24 hours after treatment commenced), confirming that at least some food was being ingested by sick animals at that time. Muscle levels in these animals were very low (0.01 mg/kg), confirming that death had occurred before effective tissue levels were reached. The OTC level of the pooled muscle sample at the end of treatment (Day 10) was 3.1 mg /kg. Muscle residues 8 days post treatment were 0.49 mg/ kg (Table 23).

No appropriate tests for tissue levels of TMZ had been validated for abalone, but as TMZ was ineffective in reducing mortality, this was not regarded as a priority and levels of the antibiotic were not measured. It had been previously shown that there was no obvious reduction in feeding with trimethoprim when used alone at these levels.

Table 23. Antibiotic residues with OTC treatment of natural *V. harveyi* infection.

Treatment Day	Sample Description	Treatment Group	OTC levels mg/kg.
Day 1 (26 Feb, 2002)	Meat only	Control, untreated	<0.05
Day 1	Gut samples	OTC treated, died	0.79
Day 1	Meat only	OTC treated, died	0.01
7 March	Meat only	OTC treated, day 10	3.1
25 March	Meat only	OTC treated, day 18 post-treatment	0.49

5.4.3.4 The effect of infection and treatments on haemocyte counts.

Haemocyte counts undertaken on randomly selected animals at the end of the treatment period (days 10 or 11) and at day 18 post treatment were 5.62 ±2.77 (n=10) and 5.53 ±2.63 (12) respectively for untreated controls; 4.13 ±3.56 (12) and 7.50 ±3.10 (12) for OTC treatment; and 5.13 ±2.95 (12) and 6.81 ±3.80 (12) for TMZ (Figure 16).

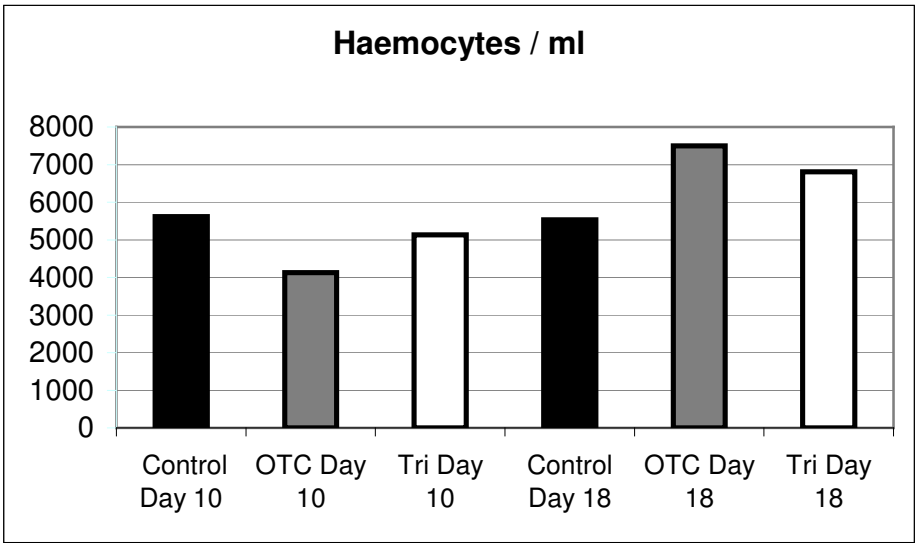

Figure 16: Haemocyte variations during treatment and recovery of a natural infection.

5.4.4 Discussion

It is an accepted principal that significant but not absolute mortality in untreated animals is a requirement for any treatment assessment, as antibiotics (or other treatments) are unlikely to be effective against overwhelming challenge. Artificial models of infection based on bacterial injection may provide a dose regime suitable for assessing antibiotic treatment, but may have little approximation to natural infection. A major advantage of injection is in providing a high level of repeatability of exposure. However for injections of bacteria this is limited by the variation between expected dose rate and the actual dose, which can only be verified retrospectively (if at all: there was failure to achieve this with the artificial infection antibiotic trial). The initial range finding experiments verified that high natural resistance is normally present in this abalone species. The series of *in vitro* tests of innate antibacterial activity suggests a high level of variability, suggesting differing levels of resistance within the population. Moreover the range-finding experiments verified the dose range in that population between an ineffective dose and full mortality was narrow, of a similar order to the variability which may occur between the nominal and actual dose. It is not therefore surprising that one dose level proved to be an insensitive infection model for antibiotic assessment. While the 50% mortality level appeared variable, it was of a similar magnitude to that found by Wang et al., 2003, with V. *parahaemolyticus* in *Haliotis diversicolor supertexta*.

It was not possible to determine directly from the second infection titration (where heat stress was applied) whether temperature stress affected the level at which death occurred, as mortalities occurred only at dilutions above those used for the first titration.

The dose rate for the artificial infection trial was clearly excessive for these animals, though the reason for differing severity between groups was not defined. The 100% mortality level indicates that failure of OTC treatment was likely to be a factor of the challenge dose. The results of *in vitro* inhibition tests from the artificial challenge trial confirm that biological

activity due to OTC was present in animals receiving OTC alone, though at a very variable level.

The few remaining animals which received bacteria and OTC showed little inhibitory activity, though the mean food consumption remained at a similar level to the controls and was only slightly reduced compared to consumption of the treatment diet by uninfected animals. While it is possible this is a reflection of the small number of remaining animals, the presence of large numbers of bacteria must also be considered. These bacteria would have been removed from the haemolymph by the filtration step, but it is unclear whether their presence would have reduced the effective levels of active antibiotic in the haemolymph during life, that is, what proportion of antibiotic is removed from the system through interaction with bacteria. Another confounding factor was the low sensitivity of this test, with untreated and seawater injected controls showing negligible inhibitory activity except with the 9:1 haemolymph to bacterial suspension. The level of inhibition in controls was considerably less than the highest levels of natural inhibitory activity which have been recorded in this system against this indicator bacteria (*V. harveyi).*

A large number of factors may interact to determine susceptibility to infection, including stress factors, and adaptation to such stress which at this stage are difficult to assess. There was a need to repeat the infectivity trial using, if necessary, multiple dose levels to ensure at least one level provided significant but not overwhelming infection. Moreover due to variations in susceptibility, the effective range may not be the same for each experiment. The opportune occurrence of natural infection in the experimental population provided such an infection.

Results from the treatment trial using natural infection did demonstrate reduced overall losses with OTC. The overall efficacy may have been greater if treated earlier, as it is possible that a large proportion of abalone carried sequestrated bacteria in locations such as abscesses by the time of treatment, into which antibiotic penetration may have been variable. It is noteworthy that a large proportion of treated abalone still showed haemolymph carriage of bacterial at the end of the treatment period, and at the end of the trial.

Antibiotic residues in this trial were carried out after separation of the muscle from gut and associated soft tissues, to eliminate the interference of residues in gut, and to give a more definitive indication of levels at the major site of action (the foot for *V. harveyi* infection). Though the peak levels cannot be directly compared with tissue levels found previously, additional samples have been held should this comparison need to be made (though beyond current budget). Results confirmed that adequate tissue levels were present within the muscle at the end of OTC treatment, and that detectable residues were still present 18 days after treated food was withdrawn. Detectable antibiotic levels in gut and in muscle even in those animals which died shortly after treatment had commenced confirmed that sick animals were still eating, and that therefore treatment would be likely to reach target animals.

No overall differences in mortality were noted with trimethoprim treatment, though some apparent reduction in mortality was noted early in the trial and the pattern of mortality was slightly altered. As only one dose rate of trimethoprim was used and this may have been relatively low in comparison with the OTC levels, which had been adjusted from the levels commonly used for freshwater fish, to at least partially off-set known interfering factors. Failure may be due to non-absorption, as absorption levels have not so far been verified due to test unavailability, but it is possible that better results might be obtained by a higher dose rate. However results do not look promising, especially as animals from the absorption trials did appear to show some tissue damage at this dose rate. If this was pursued it may also be wise to verify that delay in deaths were due to suppression without elimination of bacteria, rather than

treatment induced tissue damage. (Histology samples held should be suitable for this purpose, should subsequent studies be pursued.)

Histopathology of these three artificial infection trials provided an opportunity to define the early stages of this infection, as well as to verify that mortality was due to this pathogen. The two infection model trials showed a progressive increase in severity to day 6 with dose rate of infection up to a dose which was lethal to some of the animals. Though some of the changes were non-specific and present to a reduced degree in blank injected controls (indicating some components could reflect the injection process), this study provides a unique opportunity to confirm and describe changes associated with low levels of infection with this pathogen. As all of the high-dose animals died, and few were suitable for histopathology, this series could not be extended into the high-dose range, but by nature this is likely to have exceeded the normal intensity of infection experienced in the field. The animals studied are more relevant as the aim of the trial was to determine a moderate level that reflected the upper range of natural infection. The few animals which were examined from the higher dose tanks after death showed a range of findings that were similar to the lower doses. At low dosage bacteria were seldom detectable histologically. Abscesses were not a feature, which is not unexpected within this timeframe.

Animals from the artificial infection challenge trial extended this series. Monitoring of animals which died during the trial showed that abscesses were present within 6 days when bacteria were administered at an overwhelming dose rate. It was unclear if the slough into gut tubules seen in the single mortality in uninjected animals held at 21^{o}C was temperature related or reflected early autolytic changes. In contrast the early death in the blank injected controls appeared likely to be due to mechanical vascular disruption resulting from injection, with minor gill damage and old protein deposits in surviving blank injected animals. However contamination with the challenge organism was also a factor in this group. While the holding system was flow-through during challenge trials, an excessive bacterial load was present. AS expected from survival results, there was little difference between OTC treated and untreated infected animals, except in the single surviving animal which showed no direct evidence of remaining infection.

Of note were the animals with digestive gland granulomas. Only one animal at lower dosage showed these. While there is insufficient evidence to suggest a link with *V, harveyi* infection, the nature of these findings is of interest as such findings have been seen previously in animals from a several farms in differing Australian locations. Similar lesions have been associated with on-going but undiagnosed mortalities in at least two countries (Diggles and Oliver, 2002; Godoy and Muñoz, 2003.) Remaining autolysed animals were not examined, though they were held fixed. It is possible that granulomas could still be detected, but even if the levels in these animals was high, it would not be possible to determine whether existing an granuloma condition increased the susceptibility of these animals to *V. harveyi* type infection, or was directly associated with this.

Haemocyte counts from the artificial infection trial indicated that blank injection alone could stimulate increased circulating haemocyte count. The effect of injection was much more marked than a sudden temperature rise. This is similar to previous results showing only a slight rise with a temperature increase of this magnitude (Handlinger et al., 2004, p85).

While haemocyte the levels during natural infection were somewhat lower and more variable than previous indicative ranges established for this species (Lleonart, 2002), the overall results (in surviving animals) were remarkably stable in the untreated group over this period. Both antibiotics were associated with a di-phasic haemocyte response of a decrease by day 10, followed by increased levels at day 18. This effect was more marked in the OTC treated

animals. It is probable that the increase at day 18 reflects increased haematopoiesis initiated during the bacterial crisis.

6 GENERAL DISCUSSION

The potential for disease control using antibiotics

Achieving adequate antibiotic administration

The problems facing adequate administration of antibiotics to abalone are those common to all aquatic animals; plus specific problems associated with administration in seawater, to osmoconforming animals; and the slow rate of feeding and therefore potential for leaching and instability of the antibiotic prior to ingestion. Antibiotic administration to fish has been well reviewed extensively by Treves-Brown (2000). He demonstrated that although absorption is largely determined by the chemical, molecular weight and polar nature of the drug, there are still significant species and tissue differences with regard to absorption of different antibiotics. Absorption may also be affected by temperature, pH, and the physiological state of the animal (for example, the level of maturation in Atlantic salmon). Other relevant conclusions were that inactivation by chelation with the high levels of cations (especially Ca^{++} and Mg^{++}) in seawater, and probably in host tissues are a significant issue for some antibiotics. In general lipophilic compounds will diffuse across thin membranes such as the gill but ions with molecular weight greater than 100 will not and therefore water medication is normally recommended only for low molecular weight compounds.

In fish it is recognised that antibiotic absorption from the gut may be influenced by the amount of seawater ingested and levels of stomach acidification. This is likely to be compounded for osmoconforming invertebrates as the tissue ion levels are much higher, leaving uncertainty with regard to whether antibiotics actually absorbed remain biologically active. In addition there is recognition that in almost all diseases fish cease feeding, and that in-feed medication is therefore in general prophylactic for fish not already infected rather than therapeutic for the sick fish. Comparison of the consumption levels for the clinically normal animals used for the absorption trials and the animals exhibiting the natural infection suggest this may be less of a factor for abalone with *V. harveyi* disease. However feeding was variable, and the actual feeding rate is a major determinant of the actual dose rate, as the results above show. It is clear across all the trials that there is considerable loss of antibiotic from agar coated food, as well as reduced palatability, compared to milled-in treatments. Both leaching of the antibiotic and loss of the agar coating over the prolonged submersion period may be contributing factors. Previous research (Duis et al., 1995) had shown agar to be the preferred coating method, of those currently used. Oxytetracycline hydrochoride and amoxicillin leaching was high when applied with oil coating but reduced with agar. In that study there was no leaching of oxolinic acid from agar coating.

Bath administration is also of limited application as antibacterial agents are in general not absorbed from water by fish, with in most cases less than 5% of the administered dose being absorbed (Treves-Brown, 2000, p 58). In fish water treatment is therefore considered more suitable for treating surface infections and reducing the water load than for systemic infections. Exceptions among the available antibiotics include a few sulphonamides and the sodium salts of quinolones, which are often well absorbed. This is consistent with our findings of high absorption of oxolinic acid from bath treatment.

Of the 5 antibiotics tested, only oxytetracycline was confirmed as being adequately absorbed and clinically active with efficacy demonstrated against *V. harveyi*, the major pathogen of

immediate concern. Absorption was moderate when milled into feed, and lower for other means of application, indicating this is the application method of choice. An optimum dose for abalone has not been established. Efficacy was demonstrated using 1% active ingredient inclusion level in food, which was based on the maximum dose recommended for other animals and designed to provide an abalone dose of 200 mg/kg/day at maximum food intake. The actual dose rates achieved in the two experiments with the less then optimum actual food intake were 86 and 130 mk/kg/day, which is closer to the usual dosage used for other aquatic animals of 35-75 mg/kg/day. This will provide a guide for appropriate choice of dose rate, but as this was preliminary work, further work to establish firm guidelines may be needed before formal recommendations can be given.

Limitations on antibiotic use.

Antibiotic residue concerns

Residue clearance is a concern with OTC, due more to the rate of decline than the initial levels achieved. Known characteristics of antibiotic decline suggest decline would be exponential with the rate influenced by animal metabolic rate (that is, highly influenced by temperature in invertebrates). In these experiments, 1-week OTC residues were still more than half of day-1 levels (using Blacklip abalone held at 16-17^{o}C). Previous results from residue monitoring on Tasmanian farms following OTC treatment (nominal dose 100 mg/kg/day for 10 days, gelatin coated) at late summer temperatures showed lower initial levels as expected (3.2 mg/kg, possibly including antibiotic in gut contents). The initial rate of decline was slightly higher (0.85 mg/kg after 5 days), but residues were still nearly 0.3 mg/kg after 31 days, and 0.056 mg/kg at 61 days (unpublished results). Given that higher residue levels will take longer to decline, and that residues may decline more slowly in winter, a 90 day withholding period for OTC treatment of abalone is realistic, and may be inadequate under some circumstances, especially if no MRL has been set for abalone. This will excluding animals close to market from treatment with OTC, and add the significant cost of residue monitoring to ensure that acceptable residue levels are reached in animals marketed within several months.

Regardless of other considerations, the long residue clearance time for OTC presents a strong augment for pursuing a Minor Use Permit (MUP) for OTC use, which will result in setting of a MRL for OTC residues in abalone. Generally where no MRL has been set, the default allowable level is the limit of detection for the residue. The Australian maximum residue limit (MRL) set for salmon flesh of 0.2 mg/kg can be used as a guide to any MRL established for abalone. Allowable levels for some overseas markets could be much lower than this, and may also be taken into consideration in setting the abalone MRL.

Availability of OTC prescriptions

If OTC treatment is to continue to be used for abalone farming, the industry would need to ensure that it continues to be available. OTC is a registered veterinary chemical but currently is not registered for use in abalone. It has an S4 classification that requires that it be available only on prescription. The use of such products off-label is controlled by *the Agriculture and Veterinary Chemicals – Control of Use* legislation in each State, using national criteria. The legality of such use could therefore vary between States. Prescription off-label places the burden of responsibility for damage, including environmental damage, on the prescribing veterinarian. The willingness of veterinarians to prescribe under these conditions is uncertain, and industries are being encouraged to pursue Minor Use Permits as an alternative. OTC use in abalone would fit within the MUP guidelines (Anon, 2005). While this would involve a cost to the industry, charges and processes for MUP have recently been reviewed and revised, and as the need for these permits has increased following the implementation of the

JETACAR report processes have been simplified and charges are to be reduced. As of 1 July 2005, a flat fee of $320 will apply to minor use permits, and an assessment period of 5 months would apply (pers. com. Cheryl Javro, Aust. Pesticides and Veterinary Medicines Authority, June 2005).

Characteristics of OTC and V. harveyi infection of abalone that influence outcomes of treatment

Studies of the dynamics of *V, harveyi* infection undertaken to provide a predictable level of infection for efficacy studies suggest quite a high level of natural resistance to this disease. This is despite continued haemolymph carriage following pathogen injection, and a narrow margin between bacterial levels where no animals died and where all animals died. That bacteria survived but did not multiply to the lethal levels suggests the relevant antibacterial factors in abalone are largely bacteriostatic, rather than bactericidal. Ideally antibiotics used with abalone should therefore be bactericidal. However the most promising candidate, OTC is also bacteriostatic. This explains the bacterial carriage remaining after all treatments for the natural infection. This may also explain the narrow window between susceptibility to clinical infection and death, and the inability of OTC treatment to significantly influence the outcome of the artificial challenge trial where the dose rate clearly exceeded the lethal level for this cohort. (Equally it may indicate that treatment was sub-optimal, but antibiotic tissue levels prior to bacterial injection were shown to be adequate, though the assay of antibiotic in tissue is a measure of the reagent per se and is not a measure of bioactivity.) In all cases it is likely that the measured tissue levels are an overestimation of true bioactivity, due to chelation with cations. It is uncertain to what extent this may vary.

The bacteriostatic nature may also explain field experiences of recurrent outbreaks following apparently successful OTC treatment. Nevertheless OTC treatment of the natural infection did reduce overall losses, and it was uncertain if *V. harveyi* remained due to the dominance and possible overgrowth of sucrose positive bacteria among isolates recovered post-treatment. The efficacy may have been greater if treatment was commenced earlier, as histological examination suggested many abalone may have carried sequestrated bacteria in abscesses into which antibiotic penetration may have been poor by the time treatment commenced. Nevertheless this is likely to be a realistic scenario for any farm treatment, given the time for mortalities to reach levels of concern plus the inherent delay in diagnosis and treatment. Thus background monitoring of mortalities during at risk periods is essential to optimise antibiotic treatment.

Development of resistance rendering treatment ineffective for abalone

As well as limitations of administration and efficacy, the development of antibiotic resistance by both the target bacteria and others in the environment, and the potential for this resistance to be transferred between bacteria, is a major issue limiting the long-term availability of suitable drugs. Treves-Brown (2000) concluded that as a broad generalisation the activity of an antibacterial agent is in inverse proportion to the time it has been available.

Human safety concerns regarding transfer of resistance

Rapid potential for development of resistance, and concerns regarding the implication of resistance and antibiotic toxicity to humans, are reasons why the sulphonamides are rarely used alone, and why antibiotic use in food producing animals is restricted and likely to become more-so.

Potential for antibiotic use in abalone

The risk that antibiotic use in food producing animals pose to humans has been extensively reviewed, both nationally (JETACAR, 1999) and internationally (World Health Organisation, 1997 and 1998). There are concerns with both heritable and transferable resistance traits. Heritable antibiotic resistance develops through mutation that renders a bacterium resistant to a specific class of antimicrobials, plus selection due to antibiotic exposure. Spread of the resistance trait is vertical, but is generally not transferable to other bacteria. Mutational resistance can develop for all kinds of antimicrobials, but is especially noted for quinolones.

Transferable resistance has been described for the majority of antimicrobials, and for most disease agents is usually the most prominent type of resistance. The resistance characteristics can be spread to other bacteria through simple conjugation, though bacteriophages, or can acquire resistance genes through the uptake of naked, "free" DNA from the environment (so called transformation). Horizontal spread of antimicrobial resistance is phenomenon frequently occurring among closely related bacteria, but does occurs between distantly related bacteria, and between bacteria belonging to different ecological niches (for example hospitals and marine sediments). Resistance genes can be carried by bacteria and spread across national boundaries by movement of people, animals, feed, and food. Because various types of resistance genes can be located on the same genetic elements, horizontal spread of resistance can result in the recipient bacteria becoming resistant to several antimicrobials at the same time.

Cross-resistance, a phenomenon when resistance to one antimicrobial confers resistance to another antimicrobial, usually in the same class, is also a concern. For example, cross-resistance typically occurs between various fluoroquinolones and can also occur between different cephalosporins, penicillins, aminoglycosides and macrolides and between macrolides and lincosamides. This is a significant concern with regard to a number of antibiotics. In one study of spiked sediment (Hansen et al., 1992) oxolinic acid developed cross resistance to both oxytetracycline and flumequine, though the reverse was not true to the same extent.

International concern relates to all antibiotics, though there is particular concern with regard to the quinolones and fluorquinolones, leading to their recent withdrawal for use in food producing animals. Oxolinic acid was included in these studies as it met the criteria of use and availability for use in fin-fish, though there was doubt of the long term availability of quinolones as a group for food producing animals (JETACAR, 1999). This concern was later realised, with recommendations that approval of quinolones for food producing animals be withdrawn worldwide (Anon, 2003).

Safety to humans – occupational safety risk

Antibiotic use also poses a more immediate occupational and safety risk to workers on-farm, especially when medicated food is mixed or coated on site. In addition to of favouring resistance in opportunistic bacteria to which farm workers are exposed, repeated low levels of exposure may result in allergic reactions. Staff handling or feeding medicated feed must be made aware that they are handling medicated feed and wear appropriate protective clothing, including overalls, gloves and face mask to avoid possible allergic reactions and prevent inhalation or swallowing of medication in feed dust.

Long-term disease control options and the role of antibiotics

Overall, the opportunities for routine and sustained use of antibiotics appear limited, potentially costly, with the likelihood that treatment will not eliminate the disease from the population, and that repeated use will lead to resistance. Given the disadvantages of dependence on a single antibiotic and cost of testing any newer alternatives, efforts should

ideally be directed to alternate approaches to disease control. For this, a greater knowledge of factors affecting bacterial disease in abalone is required.

Characteristics of abalone responses which may influence the outcome of exposure

In general we have little knowledge of the number of factors which may interact to determine susceptibility to infection. It is logical to assume from other work in molluscs including abalone (Lacoste et al., 2001a; Malham et al., 2003), that variability between batches in disease susceptibility may reflect both environmental stress and previous bacterial exposure. Understanding and controlling stress is an important component of control of any disease, and the likely link between *Vibrio* related outbreaks and stress, and that prevention through stress control was preferable to emergency treatment, has been well recognised by the abalone aquaculture industry prior to this project. Improved knowledge of the dynamics and interactions of the various molluscan responses to stress is essential to improving our knowledge of susceptibility determinants which can be utilised to modify farm systems or management to avoid development of disease.

While there is limited opportunities to control stress directly related to high summer temperatures, there is a need for increased understanding of the animal and system characteristics during periods of temperature increase. This will help to better define both the primary and the secondary stressors such as decline in water quality that may be more easily controlled. Considerable progress in understanding this relationship since the start of this project (Vandepeer, 2002). Knowledge of stress responses in abalone and molluscs is also gradually improving. A number of stress related responses have recently been studied in mollusc (including abalone). They include the basic endocrine mechanisms of the response, the heat shock protein response to stress, and links between stress and susceptibility to bacterial infections (Lacoste et al., 2001a, 2001b). Specific stress research is under way in Australia (Hooper, 2004).

Haemocyte responses are one currently available measure of stress levels. The haemocyte levels recorded from the naturally infected animals are somewhat lower and more variable than previous ranges found in this species (Lleonart, 2002). There is an apparent diphasic response of a decrease, followed by increased levels, in the OTC treated animals, compared to the stable pattern for the untreated infected group, with a slight similar trend for TMP. Understanding this response is important as OTC is recognised as having immunosuppressive activity in fish: this is largely an effect on antibody production but also on monocyte mitosis, which may have parallels in molluscs. Immunological or haemocyte depletion through such means, (as has been demonstrated for immunostimulated shrimp by Lopez et al, 2003), plus the slight indications herein of tissue damage with OTC treatment, may be further constraints on dependence on this treatment.

Although haemocyte antibacterial factors are only one of the factors affecting susceptibility, their measurement during these trials clearly showed that levels of innate resistance factors active against *V harveyi* varied between different cohorts. While within-group variability was too great for the original intention of meaningful comparison of innate and antibiotic inhibition on the available number of samples, the test system developed provides a practical means to track the levels of innate resistance which is potentially an important tool for developing disease control strategies. Clearly the haemolymph antibacterial factors measured in this work are not uniformly directed to all bacteria. The number of such factors in abalone, and their degree of specificity, is not known, but this work demonstrated the need for care in selection of indicator bacteria for measurement of antibacterial factors, to ensure relevance to the pathogen or pathogens of concern. Until the characteristics targeted by antibacterial factors is better understood, the use of the pathogen of concern as an indicator may be necessary. Other characteristics of the test system, including concentration of nutrients and the number of

bacteria added may increase the sensitivity of the test and overcome the disparities seen at low dilutions.

Opportunities for alternate approaches to disease control

Tracking innate antibacterial responses could become a practical way to track exposure of animals on farms to recurrent pathogenic bacteria prior to clinical outbreaks. This may in turn assist in the development of deliberate non-lethal exposure strategies to boost immunity. At least some antibacterial activities are stimulated by exposure to dead bacteria (Li, 2000), which suggests that in the longer term vaccine like manipulation may not be impossible. If non-lethal exposure to dead bacterial products or non-pathogenic bacteria which stimulate the same factors occurs prior to rather than during periods of stress, it is likely to stimulate a much stronger response. Unlike fish, molluscs are believed not to possess immune memory (to accelerate a second response on subsequent exposure), so the usefulness of this approach would depend on the duration of this response and the ability to time this close to expected periods of risk.

Understanding the dynamics of susceptibility and response to this pathogen should enable more novel and sustainable disease control options, such as stimulation of innate immunity (that is, "vaccination") or competitive bacterial replacement (probiotics), to be developed. Empirical attempts to use stimulants without this knowledge have been largely unsuccessful and in some cases may be counterproductive. Lopez et al., (2003), found that while stimulation of the shrimp immune system with β 1-3 glucan may present with immunological fatigue following prolonged challenge, vitamin C stimulation resulted in an initially lower response but on-going immune competency. This suggests that nutritional effects on abalone defences should also be further investigated as a means of optimising abalone resistance. Chen et al, 2003, found that pyridoxine levels affected several measurable immune parameters as well as growth. Preliminary work by Boarder and Maguire, 1998, indicated that increased dietary vitamins increased both growth and survival in Australian abalone following salinity challenge, but did not identify specific vitamin requirements. The relationship of the dietary levels they tested to current diets is not known, but the possibility that levels optimum for resistance may be different to those for optimum growth may need to be further investigated.

In summary, a combination of such approaches offer more long-term hope for control of bacterial infections than routine use of one antibiotic, given the likelihood of resistance developing, the prolonged residue times with this antibiotic, and antibiotic use in food producing animals generally becoming less acceptable. Antibiotic use should perhaps be regarded as an interim and emergency measure except for specific applications such as providing withering disease free broodstock as described by Freidman et al, (2003), where treatment of a minimum number of animals, not destined for human consumption, provides an ongoing benefit to the farm. Carson, 2004, reported that *V. harveyi* pathogenic to abalone, at least from Tasmania, form a distinct group which can be differentiated with techniques now available from more common and possibly more widespread strains. Thus it is possible to test whether this is generally an abalone associated strain or more widespread in the environment. If this demonstrates a limited geographic distribution, a targeted antibiotic treatment approach similar to that used by Freidman et al, coupled with a high level of sanitation control could eliminate the infection from established farms. Such research would also indicate whether this pathogen is a translocation risk.

7 BENEFITS

The major benefit of this work was an understanding of the limitations of dependence on antibiotics for control of bacterial diseases associated with summer stress and other stresses.

The constraints highlighted include the limited antibiotic options, determined both by those which would be permitted and could realistically be registered, and by limitations on administration to slow feeding invertebrates in a marine environment.

This work also increased knowledge on innate antibacterial defences of abalone, in general and with regard to the major bacteria of concern, *Vibrio harveyi,* and provide information on factors affecting tests to measure this activity. Knowledge was also gained on infectious dose rate and the pathology associated with the early stages of this infection. The research did confirm that oxytetracycline, which has been the antibiotic most used to date, is currently effective in limiting this infection but there is long-term concern regarding development of resistance with single-antibiotic use, and long residue times.

8 FURTHER DEVELOPMENT

The major aim of assisting the process of directing future research was achieved, with advice on future research direction given throughout the project. Indications from this work were that major long-term effort should be directed to disease control through increasing understanding and control of the disease precipitating stresses. This has been translated into support for a project studying links between diet and summer mortalities, and for study of immunological and physiological changes in stress and abalone. If this information does not allow *V. harveyi* infection to be avoided, consideration should given to manipulation of innate defences to this pathogen such as development of a probiotic or a bacterin type " vaccine". The technology and capacity to achieve this does exist within Australia (J. Carson, pers com).

Confirmation that effective treatment can be achieved in the short term by OTC, coupled with the changed legislative environment for antibiotic use and the reduced costs of obtaining a Minor Use Permit, indicates that obtaining a MIP should be considered for this antibiotic.

9 PLANNED OUTCOMES

Information from this research has been extended to the Abalone Aquaculture Subprogram throughout the course of this project, and internationally to the 5th International Abalone Symposia. This has allowed the Subprogram to consider this in determining future research directions. Publication is to follow.

10 CONCLUSIONS

The potential for antibiotic us in abalone for disease control is limited, but currently possible, though oxytetracycline is still the only antibiotic with proven potential. This antibiotic was adequately absorbed from in-feed treatment and was effective *in vivo* in reducing mortalities from a natural infection with *V. harveyi*, though some bacterial carriage remained. Absorption was reduced for all antibiotics given in agar coating of food, compared to in-food treatment. Adequate absorption was not demonstrated for other antibiotics, with the exception of bath administration of oxolinic acid (which is no longer allowed). Although further work on antibiotic absorption, including antibiotics more recently registered for aquatic animals such as florfenicol, could be warranted the current work provides only one antibiotic for current use. As this situation is likely to maximise the opportunities for resistance to this antibiotic to develop, it is concluded oxytetracycline treatment be used sparingly, and that other avenues for bacterial disease control be pursued. This recommendation is difficult to reconcile with the need to treat early during a primary outbreak to prevent sequestration of the bacteria within abscesses and establishment of sustained infection within the population.

Potential for antibiotic use in abalone

Disease control through better understanding of the precipitating stresses offers better long-term potential. Other alternate control mechanisms with potential are bacterin or probiotic exposure prior to expected stress periods. The methods developed to measure the innate bacterial resistance factors offer practical ways to track these for investigation of this potential.

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12 APPENDIX 1 (Intellectual property)

No intellectual property issues associated with this project have been identified by either party.

13 APPENDIX 2 (Staff)

Handlinger, Judith	- Principal Investigator
Carson, Jeremy	- Co-investigator
Taylor, David	- Co-investigator
Harris, James	- Research Assistant
Stewart, Niall	- Research Assistant (short period)

14 APPENDIX 3 (Raw Data)

Available in electronic form.