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Bacterial Resistance To Antimicrobial Agents Used In Fish Farming; A Critical Evaluation Of Method And Meaning

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Abstract.

The use of antimicrobial agents in aquaculture has resulted in the increase in the frequency of strains resistant to these agents. Potentially these resistant strains can have an impact on the therapy of fish diseases, the therapy of human diseases or the environment of the fish farms. The analysis of the extent of these impacts is hindered by the limited information available and the variation in methods that have been used. There is, for example, considerable variation in the methods used to measure the sensitivity of strains and in the criteria used to determine the clinical significance of these laboratory data. It is important that some standardisation of sensitivity testing methods is attempted. The available data on the frequency of resistance in fish pathogens suggests that the use of antimicrobial agents in aquaculture has significantly reduced the therapeutic options available in the treatment of fish diseases. The data available to assess the impact of the use of these agents in aquaculture on the therapeutic options in the treatment of human infections is incomplete. At the present no quantitative assessment of this risk can be attempted. Considerations of the data on the impact of the veterinary use of these agents

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on the therapy of human diseases would suggest that the extent of the risk represented by their use in aquaculture is small. The epidemiology of the human pathogens that have been associated with fish would tend to confirm this assessment. There is little data pertaining to the ecology of R plasmids in the natural environment. The significance of these plasmids in transferring resistance determinants from the aquatic compartment to the human compartment can, at present, only be assessed at a theoretical level. However, such a theoretical analysis suggests that the contribution of R plasmids, selected in the aquatic environment, to the frequency of resistance in human pathogens, is probable very small. Fish farmers will have to develop methods of husbandry that limit the rate at which resistant strains emerge. Without these changes in husbandry, fish farming will rapidly enter the pre-antibiotic era. It is probable that these changes will also have the effect of reducing any impact of antimicrobial agents used in aquaculture on the environment outside the fish farm.

Keywords: Antimicrobial agents, breakpoints, environmental impact assessment, human disease, fish pathogens, R plasmids, resistance, risk assessment, selective pressure, sensitivity testing.

WHAT IS RESISTANCE?

I have yet to see any problem, however complicated, which, when looked at in the right way, did not become still more complicated.

Poul Anderson [1]

Our experience of resistance to antimicrobial agents is nearly as old as our experience of the agents themselves. Collard [2], commenting on the early work of Ehrlich and Shiga, noted that in their 1902 paper on the value of sodium arsenilate to treat trypanosomiasis, they inadvertently studied its action on a resistant strain and erroneously reported the drug as being ineffective. Despite this early introduction to the problem of resistance many recent publications addressing bacterial resistance appear to be based on a naive and simplistic definition of the term resistant when applied to a

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bacterium.

A strain of bacteria can be termed 'resistant' if it has the ability to function, survive or persist in the presence of higher concentrations of an antimicrobial agent than the members of the parental population from which it emerged. More loosely, a species can be termed resistant if its members have the ability to function, survive or persist in the presence of higher concentrations of an antimicrobial agent than the members of other species. Resistance, therefore, unlike properties such as cell shape or the ability to produce acid from glucose, is not a property that can be determined by studying a single strain. Resistance is always a relative term. It can be determined only by a comparison, under identical conditions, of the properties of two, or more, strains or species.

When in vivo resistance is the issue, not only is resistance a relative term, but also the meaning of the term is clearly context-dependent. In the context of fish therapy, the same strain may be either resistant or sensitive depending on the method by which the antimicrobial agent is administered to the infected fish, the tissue distribution of the agent compared with the location of the pathogen in the fish, and the physicochemical environment of the fish.

Under commercial farming conditions, oral administration of oxolinic acid results in serum levels of 1-2 $\mu\text{g g}^{-1}$ [3], whereas administration by i.p. injection or bath can result in serum levels of 30-40 $\mu\text{g g}^{-1}$ [4]. In a laboratory context, a strain of *Aeromonas salmonicida*, against which oxolinic acid has an MIC of 1.5 $\mu\text{g g}^{-1}$ in standard media, can be said to be resistant when compared to other strains of this species that are inhibited by 0.03 $\mu\text{g g}^{-1}$ of the same agent in the same medium. However, in a therapeutic context, this strain will be resistant if the therapy is administered orally but will be sensitive if the therapy is administered by injection or bath [5].

The chemical or physical environment of the encounter between a pathogen and an antimicrobial agent may have a significant influence on the outcome. The concentrations of Mg^{++} and Ca^{++} present in sea-water cause a dramatic reduction (>90%) in the biological activity of oxytetracycline [6] and the quinolones, flumequine and oxolinic acid [7, 8] (and unpublished results). The concentrations of these ions in the hind gut of a fish in sea water may be even higher than those found in the water itself [9]. Thus, a strain that encounters these antibiotics in the hind gut of a fish may be sensitive

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or resistant depending on whether the fish is in sea water or fresh water. The physical nature of the environment may also play a role in determining the clinical outcome of a therapy. In *in vitro* tests, the MIC of oxolinic acid and of oxytetracycline against a strain depends on the temperature of the test environment [10-12]. Temperature also has an effect on the concentration of an agent that is achieved in a fish following a standard administration protocol [13, 14]. Fish are poikilothermic and, therefore, therapy may occur over a wide range of internal temperatures. Thus, the clinical sensitivity or resistance of a strain may depend on the temperature of the host.

In the context of an infection, the sensitivity or resistance of a strain may be a function of the location of the pathogen and the tissue distribution of the antimicrobial agent. The best illustration of this can be drawn from human medicine. Oral therapy of humans with flumequine typically results in levels of 10-13 $\mu\text{g g}^{-1}$ in serum and 200 $\mu\text{g g}^{-1}$ in the urine [15]. Thus, the same strain may be resistant if it is in the blood of a human patient but sensitive if located in the urinary tract.

Under commercial farming conditions, the majority of therapeutic treatments are administered orally to fish. Loss of appetite, which is a common symptom of infection in fish, introduces further complications to the problem of defining resistance. The food consumption of an individual fish may play a role in determining the sensitivity or resistance of a pathogen to a particular therapeutic treatment. A pathogen may be sensitive to the concentrations of an agent achieved in a fish that is feeding well but may be resistant to the concentrations that are achieved in a fish with reduced appetite.

It is clear that the terms resistant or sensitive can not be considered as being absolute, objective properties of a particular strain. Each time these terms are used, they must refer to a specific context. This emphasis on the context-dependent nature of the meaning of the term resistant is not purely a pedantic obsession with words. It has important practical implications. In any study of bacterial 'resistance', the specification of the context, providing meaning for the use of the term 'resistance', must play a major role in determining, particularly in their quantitative aspect, the methods to be used. These implications will be discussed in more detail in the following sections.

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IMPACT OF ANTIMICROBIAL AGENT USE IN FISH FARMS ON THE FREQUENCIES OF RESISTANCE IN FISH PATHOGENS

The scientific mind does not so much provide the right answers as ask the right questions.

Claude Levi-Strauss [1]

It is a general, though far from absolute rule, that the more often an antimicrobial agent is used in an environment, the higher will be the frequency of resistant microorganisms in that environment [15]. The ability of strains of pathogens to emerge that are resistant to clinically-achievable concentrations of antimicrobial agents represents a major factor in limiting the value of these agents. It is, therefore, reasonable to expect that a major effect of the use of antimicrobial agents in fish farming will have been an increase in the frequency of resistance in fish pathogens. In attempting to find evidence to support this assumption and to estimate the extent of the phenomenon, a major problem is immediately apparent in the published literature; methodological chaos. Not only is there little agreement as to how resistance should be defined, there is not even any consensus as to how it should be measured. A survey of 23 papers, that present information regarding the sensitivity of fish pathogens to antimicrobial agents, revealed that 7 gave incomplete methods and, of the remaining 16 papers, a total of 10 different methods were used. In 6 of these 23 papers, no information was given on the experimental definition of the term 'resistant'. A total of 9 different definitions were used in the other 17 papers [7, 11, 16-36]. The recent report [37] of a comparative study of sensitivity testing in six countries demonstrates that these differences in method and definition have important practical implications. There was significant disagreement between the laboratories as to whether the same strains should be classified as sensitive or resistant. It is, therefore, clearly important to discuss the laboratory methods available for measuring sensitivity, the interpretation of the data these methods generate, and the implications of the lack of consistency of method between different laboratories.

Laboratory Methods for determining sensitivity.

A variety of laboratory tests can be used to generate sensitivity data. Ultimately, the most important property of any in vitro sensitivity test is that the results it produces

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can be used to generate reliable predictions of efficiency of therapeutic treatments in vivo. It is important to recognise that there will necessarily be differences between the environment of the pathogen during the in vitro laboratory test and the environment it experiences in vivo. These environmental differences may result in significant differences in the concentrations of antimicrobial agents required to inhibit or kill the pathogens. Laboratory media are not formulated to simulate in vivo conditions either with respect to their pH, nutrient conditions or ionic concentrations. Brown [38] has argued that in the host, many aspects of the phenotype and importantly the sensitivity of pathogens to antimicrobial agents will be influenced by phosphate limitation, yet laboratory media are frequently buffered by relatively high concentrations of phosphates. Similar arguments can be made about the availability of iron in laboratory media. Gilbert and Brown [39] have argued that it is possible that many of the elements of laboratory sensitivity-testing protocols, such as centrifugation of inocula, that were included in order to increase the reproducibility of the tests, may have inadvertently increased the differences between in vitro and in vivo sensitivities. A variety of other factors may also introduce complications into the comparison of in vitro and in vivo sensitivities. Host-defence mechanisms play a significant role in the outcome of infections and a variety of interactions between antimicrobial agents and these mechanisms have been reported [40]. Sub-inhibitory concentrations of antimicrobial agents may also play an important role in defending the host. These concentrations have been shown to alter the adhesive properties of pathogens [41] and, in a phenomenon termed post-antibiotic effect [42, 43], have been shown to kill cells previously exposed to higher levels of the agent. On the other hand, sub-inhibitory concentrations may also select, at relatively high frequencies, variants manifesting persistence mechanisms which result in a phenotype with reduced sensitivity to the selective agent [35, 44].

Having considered these problems, the British Society for Antimicrobial Chemotherapy (BSAC) working party on sensitivity testing [45] has suggested that the two aims of laboratory reproducibility and clinical relevance may be fundamentally irreconcilable. If this is the case, then the degree of inter- and intra-laboratory reproducibility that can be achieved using a particular in vitro method may be more important than the relevance of its results to the in vivo situation. The majority of in vitro

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sensitivity tests on fish pathogens have been performed either by disc diffusion or dilution methods. New, more rapid, methods have been reported using either immunological [21], conductance [46], or DNA probe [47, 48] technologies. As these have yet to be used widely in the study of fish pathogens, they will not be discussed further in this review.

Disc diffusion methods

Disc diffusion assays are frequently the method of choice in diagnostic laboratories where information is required rapidly on the sensitivity of a limited number of isolates to a range of antibiotics [49]. The size of the inhibition zone produced in such tests is a function of the method used to determine it. Cooper [50] has shown that, in addition to the sensitivity of the organism and the amount of the agent used, the diffusion rate of the agent and the growth rate of the organism are primary factors in determining the size of a zone. When performing sensitivity tests using a disc diffusion test, it is vital that the composition, pH, humidity and volume of the medium, the incubation temperature and the method of production of the inoculum, and the size and method of inoculation are standardized [45]. A comparative study of the disc diffusion methods currently used in fish disease laboratories in six countries has been co-ordinated recently by Olivier [37]. Considerable differences in all parameters of the disc diffusion test were noted between the methods employed in the six laboratories. There were significant variations in the zone sizes recorded for a set of 10 strains even between those laboratories using the same amount of agent in their discs. These variations were presumably a function of the variations in other parameters of the method. Possibly of greater practical significance than the variations in the zone sizes was the extent to which the laboratories disagreed as to whether the strains should be classified as sensitive, intermediate or resistant. In this respect, the greatest disagreement occurred in reporting sensitivities to the quinolones, and the potentiated sulphonamides.

The results of disc diffusion assays are normally recorded as the diameters of the zones of inhibition produced. However, these diameters are related to MIC values. With many, but not all antimicrobial agents, a linear relationship exists between the diameter of the zone of inhibition produced using a standard method, and the log of the MIC of the

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agent against the organism [51, 52]. Where this relationship holds, it is possible to deduce the MIC of an isolate from a regression line produced by performing a large number of MIC and disc diffusion tests simultaneously. At the time of the writing of this review no regression lines for fish pathogens had been published.

Several standard disc diffusion methods have been developed for use with human pathogens [49]. Some require detailed internal comparative standards to compensate for variations in reagent quality [53], whereas others rely on rigorous specification of method [52, 54]. With the increased standardization of laboratory reagent quality, it has been suggested that the second group are now more suitable [55]. However, it should be noted that all methods require the use of standard control organisms. Methods developed for human pathogens require modification before they can be applied to fish pathogens. Notably, the choice of incubation temperatures and times, and that of the standard control organism to be used will be different. These necessary modifications will result in an alteration of the regression line relating zone size and MIC for a particular agent and therefore alter the relationship between zone size and breakpoint concentration (see below). In studies of fish pathogens, the most frequently used disc diffusion methods have been modifications of the Kirby Bauer method [54]. It should be noted that this method is dependent on the existence of standard regression lines for the interpretation of the results it generates.

Dilution methods

The most common alternatives to disc diffusion assays are the dilution methods using either solid or liquid media. These are more time-consuming, but have the apparent advantage in that they generate numerical values for the MICs directly. In cases where the sensitivity of a strain is in doubt, determination of the MIC by dilution methods is generally perceived as being the more 'correct' approach [45]. Atkinson [56] has reported a general trend toward a more widespread use of dilution methods in laboratories examining human pathogens. Broth dilution methods have an additional advantage in that they can be adapted to determine MBC values [35]. It is important to note that the use of solid or liquid media may generate numerically different MIC values [52]. Liquid cultures inhibited by the quinolone antibiotics may continue to increase in optical density

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as a consequence of cell filamentation. For this reason broth dilution methods are not appropriate for testing sensitivities to the quinolones [22]. Disc diffusion assays are critically dependent on the growth rate of the strain under test and dilution methods are more appropriate when the sensitivities of slow growing strains are to be determined [45]. Logistical problems associated with the dilution methods have resulted in their being used most frequently when a large number of strains are to be examined at the same time. The development of microtitre dilution methods reduces the logistical problems associated with these methods [24]. Consequently, this may lead to the more frequent use of these methods in diagnostic laboratories. The need for standardization of the media, inocula and incubation conditions for dilution methods is just as great as it is for diffusion methods. The 10-fold variation in the published numerical values of the MIC of oxytetracycline against fully sensitive strains of *A. salmonicida* amply illustrates this point [10, 22, 30, 35, 57].

Interpretation of laboratory data

Data generated by laboratory sensitivity tests, by which ever method, must be related to probable clinical efficacy of any therapy. Essentially, critical breakpoint values of the parameter measured in the laboratory must be established, so that strains tested can be placed in one of the clinically relevant categories i.e. sensitive, resistant or intermediate. In some studies of fish pathogens these critical values have been established by analysis of laboratory data alone. This approach has been taken, for example, by Aoki et al. [58, 59] and Kusada et al. [27, 28]. The laboratory test, dilution MIC or disc diffusion, is performed on a large number of strains of a particular species. Provided that these values show bimodal or polymodal distributions, the data can then be used to establish breakpoint values to discriminate between sensitive, resistant and intermediate strains. The determination of breakpoint concentrations from MIC data alone takes no account of the pharmacokinetics of the agents or clinical experience of therapies. In human medicine, the published breakpoints have been determined using combinations of data from all three sources breakpoints [45, 60, 61].

Pharmacokinetic data

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The BSAC [45] has recommended that the determination of breakpoint concentrations should be based on comparisons of laboratory-determined values for the MIC of a strain with the concentration of the therapeutic agent that is achieved in the host. Superficially, this approach has the apparent advantage in that the necessary data for the establishment of the critical breakpoint values can all be measured accurately. However, in practice, the application of this approach has been fraught with methodological problems. These problems have been addressed in the study of therapy of human diseases but have received little sophisticated attention in fish diseases beyond the assumption that the serum MIC should exceed the in vitro MIC by a factor of 3-4 [57]. The central methodological problems concerning the measurement of the concentration of antimicrobial agents in fish are; where in the fish should concentrations be determined, by what method, and over what time period should they be determined?

The location in the host fish where the concentration of the agent should be determined will depend on the nature of the infection. The ease with which blood samples can be collected has resulted in serum or plasma concentrations being the most frequently reported data, but these may have limited relevance in some infections. In the early stages of carp erythrodermatitis, for example, the pathogen is confined to skin lesions [62]. Similarly, limited distribution of the pathogen has been observed in summer lesion syndrome, a condition that has been the cause of considerable antibiotic use in Irish marine salmon farms (unpublished data). It is possible that muscle, or skin concentrations would provide more reliable data for predicting clinical efficiency of treatments of these conditions. If Markwardt and Klontz [63] are correct in suggesting that the intestine is the primary location of the pathogen in asymptomatic furunculosis infections, then concentrations achieved here may be of greater significance than serum concentrations in predicting the efficacy of any treatment aimed at eliminating such infections. Even in the case of a disease where there is unambiguous evidence of systemic distribution of the pathogen [62], it is not certain that serum concentrations are the most relevant data. Therapeutic treatments in fish farming are primarily aimed at controlling epizootics rather than the infections of individual fish. Loss of appetite is frequently observed in those fish in a population that has a systemic bacterial infection. A consequence of this inappetance is that oral therapy of a fish population will frequently

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deliver the agent to only those fish that have yet to be infected. The treatment is, therefore, prophylactic rather than therapeutic. The aim of prophylactic therapy is to prevent the initiation of an infection rather than to cure an existing infection. It is possible, therefore, that antimicrobial agent concentrations at the, frequently unknown, site of entry of the pathogen into a new host, may be highly significant in prevention of epizootics.

In determining clinical efficiency, the critical parameter is the concentration of biologically-active agents that is achieved in the treated animal. A variety of parameters, including the extent of protein binding and the ambient ionic concentrations, may result in reduced biological activity of an antimicrobial agent in the internal environment of a fish. There is only limited data available on the extent of protein binding in fish [64, 65]. The majority of measurements of antimicrobial agent concentrations in fish have been made using HPLC techniques. These measure both accurately and precisely the chemical concentration of the agents *in vivo*, but are incapable of determining biological activity. If data generated by HPLC or other chemical detection measurements are to be used, then they must be modified by conversion factors designed to compensate for inactivation resulting from the chemical nature of the *in vivo* environment [45]. These considerations might suggest that biologically-based assays of *in vivo* concentrations may be more suitable. It should be noted, however, that most recommended bioassay methods for the detection of antimicrobial agents in serum recommend that test samples should be compared with standards made up in the same serum [66, 67]. These protocols, therefore, result in bioassays that attempt to measure chemical concentrations rather than biological activity. The use of standards made up in defined buffers might, in this situation, generate data that is more relevant and more immediately applicable.

The action of an antimicrobial agent against a pathogen is time-dependent. It is therefore necessary to decide over what period the concentration of the agent in the host should be determined. Lambert and O'Grady [15] have suggested that the concentration that is exceeded for 50% of the time between doses may be a useful figure. On the other hand, the BSAC working party [45] has suggested that the C_{\max} could be used, if it was modified by a factor related to the half-life.

Under farming conditions antimicrobial agents are presented orally over a number

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of days, often to populations of diseased fish with significantly reduced food consumption. There are few studies of the concentrations achieved under these conditions [3]. There are some relevant data in studies of tissue withdrawal kinetics. These have recently been reviewed by Ellis [68]. Other data have been generated in single dose pharmacokinetic studies. The most relevant of these are those produced following the oral administration of agents in feed [14, 64, 69-73]. The extent of variation in experimental design and in the concentrations detected, limits the value of these data in the formulation of breakpoint values. The data do, however underline the importance of temperature in determining antimicrobial agent concentrations achieved in fish [13, 14, 74].

It is clear that the methodological problems involved in establishing breakpoint concentrations from pharmacokinetic data are numerous and, possibly intractable. Thus, precise determinations of breakpoints cannot be made using data from accurate laboratory measurements alone. In human medicine, breakpoints have been defined primarily using pharmacokinetic data [45, 60] or MIC distributions. However, in both cases, it has been found necessary to include some factor representing clinical experience in the final determination of breakpoints.

Clinical experience.

The relationship between MIC values and clinical efficacy in the treatment of fish diseases has been little studied and is difficult to determine with any degree of confidence. Studies have been published on the efficacy of certain chemotherapeutic treatments. However, in fifteen randomly selected papers on this topic, over half presented no laboratory data on the sensitivity of the pathogen used in their studies. In the treatment of fish diseases, assessments of efficacy are complicated by a number of factors such as the feeding response of the fish, the timing of the start of therapy, the presence of unrelated stress factors and the economic and ethical factors that proscribe the use of untreated control groups. With the exception of one attempt to establish correlations between the clinical efficacy of therapeutic treatments of furunculosis with quinolones and laboratory sensitivity data [5] there is little published information available in this area. Many veterinarians and fishery biologists have acquired considerable experience in

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making therapeutic decisions based on laboratory data. It might be thought that collating these experiences might provide a set of data that would facilitate the determining of relevant breakpoint values. The failure of research laboratories to establish standard methods for sensitivity testing has the unfortunate consequence of limiting the potential value of this exercise.

Breakpoints in practice

Given the difficulty in relating laboratory data to the clinical situation, some authors have questioned whether sensitivity testing is of any value in guiding therapeutic decisions in human [75, 76], or veterinary [77] medicine. It should be noted that although difficulties are theoretically present in all laboratory assessments of clinical sensitivity, ambiguities of practical significance are encountered only in a minority of cases. Phillips [78] has argued that, for organisms exhibiting a widely separated bimodal distribution of MICs and for which the MICs of sensitive strains are low, predictions of the clinical outcome can be made with a high degree of reliability. In the main, problems arise only when strains of intermediate sensitivity are encountered. Unfortunately, strains classified as being of intermediate sensitivity are not rare in studies of fish pathogens. For example, Høie et al. [24] using the breakpoints of Tsoumas et al. [35] classified 22% of the 138 strains of *A. salmonicida* he examined as being of intermediate sensitivity to potentiated sulphadiazine. Tsoumas et al. themselves were forced to classify 35% of their 70 *A. salmonicida* strains as being of intermediate sensitivity to the same agent. Schlotfeldt et al. [79] did not specify the breakpoint values they used but with respect to oxytetracycline they placed approximately 35% of 600 strains in an intermediate category. From a clinician's point of view, the classification of an isolate as being of intermediate sensitivity is of little value. Prudence would suggest that such strains have to be treated, possibly erroneously, as being resistant.

In practice, if sensitivity tests are carried out, all diagnostic laboratories must use some breakpoint concentrations in interpreting laboratory data. There has been a lack of consistency in those applied in the study of fish pathogens. Waltman and Schotts [36] have applied the breakpoints recommended by the National Committee for Clinical Laboratory Standards [61] to laboratory data on fish pathogens. The relevance of these

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breakpoints is limited by the fact that they were established for use with human pathogens and for the interpretation of data from MIC or disc diffusion assays designed for use with these strains. Aoki et al. [18-20] have based their interpretations of laboratory data on the distribution of MIC values as determined by the methods of the Japan Society of Chemotherapy [80]. Tsoumas et al. [35] published suggested breakpoint MIC values that had been determined following consideration of a number of parameters including MIC values pharmacokinetics and clinical experience. These values have subsequently been applied by Høie et al. [24]. Inglis et al. [11] have published breakpoints in terms of zone sizes.

Irrespective of the parameters and calculations used to determine the numerical value of breakpoint concentrations, their predictive value is critically dependent on the use of a specified standard method to generate the laboratory data. Their value in clinical prediction would be seriously decreased if they were to be applied to data generated by a method other than that used to establish them. The importance of this point can be illustrated by reference to the breakpoint of 1.0µg ml⁻¹ of oxytetracycline suggested by Tsoumas et al. [35] as the upper limit for strains of *A. salmonicida* considered to be fully sensitive. In their laboratory the median MIC values for sensitive strains was 0.125 µg ml⁻¹. The use of their breakpoint values by workers using laboratory methods which give median MIC values of 1.0µg ml⁻¹ for the sensitive strains [10, 22, 30, 57], would clearly be inappropriate and may easily lead to the giving of misleading therapeutic advice.

Multiple low-level resistance, persistence and transitory resistance mechanisms

Two, possibly related, aspects of resistance, multiple low-level resistance and transient persistence mechanisms also merit mention. Strains of *A. salmonicida* selected, in the laboratory, for low-level increases in resistance to one antibiotic may show a phenotype that manifests low-level resistance (MLLR) to a range of other antibiotics [81, 82]. This MLLR phenotype has been reported in many other genera [83]. In *A. salmonicida* the MLLR phenotype has been shown to arise as a result of changes in membrane proteins [81, 84, 85]. Barnes et al. [84] have also demonstrated the MLLR phenomenon in field isolates. The levels of resistance detected were such that the strains would be classified as being of intermediate sensitivity using the breakpoint values of

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Tsoumas et al. [35].

Bryan [44] has drawn attention to the importance and significance of a phenomenon he terms 'persistence' in understanding the resistance of pathogens to chemotherapy. Persistence mechanisms, which may result from genotypic or phenotypic changes in the cell, are characterized as those that become apparent only in the presence of the antimicrobial agent. Thus, they will be detected only during, and for a variable but limited time, after therapy. Although, by this definition, persistence mechanisms must be transient, it is important to note that this refers to the survival of the resistant strain in the natural environment. This transience may not necessarily be manifest in pure cultures held under laboratory conditions. For example, strains whose persistence mechanism is associated with a slight reduction in growth rate will be rapidly outgrown and therefore become transient in the natural environment but may well fail to manifest this transience in the laboratory. Bryan [44] identified a number of antimicrobial agents to which elevated resistance has been shown to be mediated by persistence mechanisms. Of particular interest is his observation that, in the case of the quinolone antibiotics, persistence was probably the dominant mechanism of clinical resistance. In this context, however, it should be noted that strains of *A. salmonicida* exhibiting increased resistance to oxolinic acid have become endemic in some Irish farms and have continued to be isolated years after the last use of any quinolones (unpublished results). Thus, the elevated resistance to the quinolones in these strains has shown no signs of transience in the environment.

Persistence mechanisms may have an important impact on the selection of a suitable therapeutic agent with which to treat an epizootic in fish. Persistence can frequently be mediated by changes in the permeability of the cell membrane and the decrease in sensitivity produced by such changes may be relatively small [81, 84]. It has been argued above that strains that are neither fully resistant nor fully sensitive present the biggest problems for a clinician in interpreting laboratory data. Persistence mechanisms may be a significant method by which such strains of intermediate sensitivity are generated. A second problem is associated with the fact that persistence mechanisms develop only during therapy. It is normal practice to determine the agent to be used in treating an epizootic on the basis of sensitivity tests carried out prior to the

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initiation of treatment. The development of persistence during an epizootic may result in these initial sensitivity tests becoming a misleading indicator of the value of a therapeutic regime later in the course of the epizootic.

Persistence may also present problems in retrospective surveys of the frequency of resistance in laboratory collections. The decreased sensitivity of some strains may be a function of a persistence mechanism that is not stable in laboratory culture. Thus, in the absence of continuous selective pressure, the phenotypes of these strains may revert to wild type. Equally, it should be noted, that storage of strains under selection pressure may also produce changes in the levels of resistance. This instability of resistance phenotypes has been observed in our strain collection in Galway, and has been noted most frequently in the levels of resistance to oxolinic acid and flumequine.

There have been no studies aimed specifically at investigating the frequency of the emergence of strains with persistence mechanisms during the therapy of fish epizootics. However, *in vitro* experiments suggest that such a phenomenon is not improbable. Exposure of a fully sensitive strain of *A. salmonicida* to concentrations of oxolinic acid, oxytetracycline and potentiated sulphonamides that were half the MIC, was shown to result, at a high frequency, in increases in resistance. These increases in resistance could be repeated, and after 15 such exposures the resistance level had risen 20-fold, 16-fold and 16-fold respectively for the three agents [35, 86]. During subsequent culture in the absence of these agents the increased resistance to oxytetracycline and the potentiated sulphonamide, but not oxolinic acid, were shown to be unstable. A further illustration of the unexpected problems that persistence mechanisms may pose comes from the unpublished results of tube dilution determinations of the MIC of flumequine against *A. salmonicida* in our laboratory in Galway. These results also suggest a link between MLLR and persistence. In these experiments the total inoculum per culture tube was 5×10^5 cfu. However, following incubation, 100% (n=10) of the colonies isolated from the highest dilution in which growth occurred were shown to have an 8-fold higher resistance to flumequine than the strain used to inoculate the tubes 72 hours earlier. All of these strains also exhibited a significant decrease in their sensitivity to oxytetracycline.

Some field observations also suggest that the MLLR phenomenon may be mediated by a persistence mechanism. A strain of *A. salmonicida* with a slightly reduced

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sensitivity to oxolinic acid (MIC 0.5µg ml⁻¹) was isolated from a marine salmon farm during an epizootic of furunculosis. The outbreak was treated, unsuccessfully, with oxolinic acid. Isolates obtained during the period of therapy showed a further decrease in sensitivity (MIC 5µg ml⁻¹). Laboratory tests showed that this strain, in addition to its decreased sensitivity to oxolinic acid, also exhibited decreased sensitivity to other quinolones, oxytetracycline, amoxycillin, ampicillin and chloramphenicol but not to the potentiated sulphonamides or streptomycin. Two lines of evidence indicate that the MLLR phenotype was transient. Furunculosis became an endemic problem on the farm but all subsequent isolates manifested an MIC of 0.5µg ml⁻¹. In the laboratory this MLLR phenotype was also lost following prolonged storage at 4°C. Thus, the MLLR phenotype exhibited all the properties expected of a persistence mechanism.

Taken together, these data on MLLR and persistence mechanisms raise a frightening prospect. They suggest that the use of an antimicrobial agent may result in the rapid development, during therapy, of strains with decreased sensitivity, not only towards the agent being used, but also to a significant percentage of alternative agents that could be used. Although it is clear that further research is required to determine the frequency with which this phenomenon actually does occur under farming conditions, any fears should be tempered by the knowledge that the therapeutic use of the quinolones has, in many epizootics, exerted effective control of mortalities [87].

Resistance in fish pathogens

Reports of the frequency of resistance in fish pathogens have been published in a number of studies. The design and aims of these studies have been varied. Some have been concerned with the evaluation of new antimicrobial agents, some with the frequency of R factor-encoded resistances, and still others with the frequency of clinical resistance. Variations in both laboratory methods and in the criteria for the designation 'resistant' preclude detailed comparisons of these data. Further, in many studies, the set of strains examined were not selected on stringent epidemiological grounds, rather they were derived from samples submitted to a central laboratory. Thus, the strains examined are frequently not truly representative of the area, industry or country being studied. Significant biases may have been introduced by the over-representation of strains from

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problem epizootics or of strains from a particularly vigilant veterinarian or fishery biologist. The observation [11, 87, 88] that strains with more than one antimicrobial sensitivity pattern are often isolated from epizootics also complicates the collection of a representative strain set. The variation and lack of definition in the methods used to select strains, even within one country, present difficulties in comparing changes in the frequency of resistance in a pathogen or pathogens over time. The problems that may arise can be illustrated by data collected in our laboratory in Galway. Resistance to oxolinic acid rose from zero to 78% within one year of the introduction of the agent (unpublished results). Epidemiological analysis of this event revealed that these strains had, in the main, been isolated from seven sea farms, and that these farms had all been supplied with smolts from a single hatchery. The apparent dramatic rise, in the frequency of oxolinic acid resistance, was largely the result of multiple isolations of a single resistant strain that originally emerged in this hatchery.

Despite these reservations about the quality of the available data it is possible to detect three major trends in the frequency of resistance in fish pathogens. Firstly, nearly all studies show high levels of resistance in these pathogens. Secondly, when data on the use of therapeutic agents are available, it is clear that the prevalence of resistance to specific agents reflects the frequency of the use of the agent. Thirdly, R plasmid-encoded resistances are common in fish pathogens.

Table 1. Reported frequencies of antimicrobial agent resistance in fish pathogens isolated in Norway and Scotland.

Country	Species	Strains	Date	Resistance (%)				Ref
				OTC	OA	PSA	All 3	
Norway	V. salmonicida	74	1986-7	54	nr	45	nr	89
Norway	A. salmonicida	138	1991	18	30	14	8	24
Scotland	A. salmonicida	122	1988-9	55	41	11	4	87
Scotland	A. salmonicida	144	1989-90	55	31	10	6	87
Scotland	A. salmonicida	178	1990-1	50	54	14	12	87

nr = not reported, OTC = oxytetracycline, OA = oxolinic acid, PSA = potentiated sulphonamides

Frequency of resistance in fish pathogens and its relation to antimicrobial agent use.

In order to analyse the frequency of resistance of fish pathogens, only those studies where it is probable that the strain set examined was reasonably representative,

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will be discussed. Table 1 presents a summary of the data available on the frequency of resistance in *A. salmonicida* and *Vibrio salmonicida* strains isolated from salmon farms in Europe. Additional data on the resistance patterns of *A. salmonicida* in Norway, not included in Table 1, has also been presented by Hopp and Olsen [88]. It is clear from these data, that in many epizootics of this disease, the choice of therapeutic agent is limited by the resistances of the etiological agent [24, 87]. Although no quantitative data is presented, the authors of these papers, all report that the frequency of resistance reflects the pattern of antimicrobial agent usage. The nature of the data available from Norway makes it difficult to assess any increase or decrease in the frequencies of resistance over time. The report of a reduction in the overall amount of antibiotics used in Norwegian fish farms during the late 1980's should be treated with some caution [90]. The reduction in amount coincided with a change from the use of oxytetracycline to the use of oxolinic acid. This, in itself, would have produced a reduction in the total amount of antimicrobial agents used without any decrease in the number of therapeutic treatments. The Scottish data from 1988-1991 [87] shows little evidence of an increase in the frequency of resistance to individual agents but shows a definite increase in the frequency of multiple-resistant strains. Meier et al. [91] and Schlotfeldt et al. [79] have published data on the frequencies of resistance in isolates of specific and facultative fish pathogens of fresh water fish isolated in Switzerland and Germany respectively. Both authors report significant and increasing resistance frequencies in most bacterial groups. Table 2 presents a summary of data on the resistances detected in isolates of a variety of pathogens collected in Japan. Again, it is clear that resistance of these pathogens frequently places constraints on the choice of therapeutic agent that can be used. There is, in these papers, a lack of quantitative data on the total amount of the antimicrobial agents used in Japan or their relative importance. Many authors, however, comment that the patterns of resistance reflect the prevalence of the use of these agents [18, 19, 59, 92-93, 95-97]. The studies on the frequency of resistances in *Pasturella piscicida* between 1984 and 1988 demonstrate, for example, the increase in the frequency of resistance to the quinolone antibiotic during this period (Table 2). Of equal importance is the observation that, in some cases, a reduction in frequency of resistance has been detected following the reduction in the use of a particular agent [18, 59, 93]. A similar comment has been made

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concerning the frequency of oxytetracycline resistance in Norwegian salmon farms.

Table 2. Reported frequencies of antimicrobial agent resistance in fish pathogens isolated in Japan.

Species	Strain	Year	Resistance (%)							Ref
			CMP	OTC	TMP	AMP	NF	NA	OA	
<i>P. piscicida</i>	281	1981-83	59	59	0	15	67	8	nr	92
	307	1984	99	84	nr	49	nr	13	13	27
		1985	99	99	nr	34	nr	25	25	27
	306	1986	nr	nr	nr	35	nr	nr	34	28
		1987	nr	nr	nr	34	nr	nr	34	28
		1988	nr	nr	nr	37	nr	nr	40	28
<i>E. tarda</i>	168	1972-79	32	0	0	12	74	15	nr	19
<i>V. anguillarum</i>	256	1974-7	65	34	34	0	94	88	44	18
	65	1978	10	0	0	0	63	78	nr	93
	45	1979	53	0	0	0	76	89	nr	93
	114	1980	54	39	1	49	73	78	nr	93
	139	1981-3	42	25	80	51	54	60	nr	59
	114	1989-91	18	18	18	91	81	75	nr	94
<i>A. salmonicida</i>	129	1979-81	14	4	2	1	92	47	48	95

nr = not reported, CMP = chloramphenicol, OTC = oxytetracycline, TMP = trimethoprim, AMP = ampicillin, NF = nitrofurazolidone,

In the US, studies involving a limited number of strains demonstrated R plasmid-encoded resistance to oxytetracycline in *Aeromonas hydrophila* (5/24) and *A. salmonicida* (4/12) isolates [30]. In contrast, very low levels of resistance were reported in 118 strains of *Edwardsiella ictaluri* isolated from catfish in the south-eastern US between 1977 and 1984 [36]. More recent reports of McPhearson [29] and DePaola [98] on the frequency of resistance in the microflora of the effluent of catfish farms would suggest that the frequency of resistance in this area may have increased.

The significance of R plasmids in resistant fish pathogens.

Numerous authors have reported the presence of R plasmid-encoded resistance determinants in fish pathogens [17, 19, 20, 30, 34, 92, 93, 96-107]. A limited number of recipients, normally one, were used in these studies. As a consequence, the reported frequencies are necessarily, underestimates of the total R-plasmid occurrence. A high

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degree of similarity at the level of incompatibility group and restriction fragment length polymorphism has been reported in R plasmids detected in strains of the same species isolated from a number of different farms and also in those detected in pathogens of different species [19, 59, 92, 93, 99, 104, 105]. The vast majority of the R plasmids identified have been classified as being members of incompatibility groups C and U. The group Inc A-C is frequently referred to by Japanese workers [108]. In this review we follow the classification used by the UK Public Health Laboratory, Colindale, which includes Inc A-C plasmids as part of the Inc C group [109, 110]. The fact that two classes of R-plasmids are dominant amongst fish pathogens may be of general significance for our understanding of R-plasmid ecology.

The only observation of R plasmid acquisition by a fish pathogen, as a result of therapy, is that of Brazil et al. [111]. However, it is highly probable that under the selective pressure exerted by therapy, R plasmids have a significant role in the spread of resistance determinants amongst fish pathogens. The data in Table 2 demonstrates that the frequency of resistances to oxolinic acid, which is not plasmid-encoded [112], can also increase rapidly. Thus, significant and rapid increases in the frequency of resistance in fish pathogens, is not confined to those resistances encoded on R plasmids.

POTENTIAL THREAT TO HUMAN THERAPY.

Scientific research has become so complicated and demands such enormous apparatus that only the State and immensely rich patrons can pay for it, which in practice means that a disinterested search for knowledge is cramped by the demand for results that will justify the expense: the scientist must turn showman.

Robert Graves [113]

The demonstration of transferable drug resistance plasmids (R plasmids) in bacteria [114] raised the possibility that the unlimited, epidemic spread of these plasmids through pathogenic species might seriously limit the value of these agents in disease therapy. The rapid increase in the frequency of R plasmids in *Shigella* strains, isolated in Japan in the early 1960's [115], provided evidence to support this hypothesis. Concern was expressed that veterinary use of antibiotics [116, 117, including their use in fish

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culture [118] could increase both the selection of resistant strains and their spread to strains of importance in human disease. Twenty years later, these issues have still not been resolved [119, 120]. Bywater and Verschuere [121] have suggested that they remain an area of controversy where fact and emotion can too easily become intertwined. This mixture is commonly encountered in environmental impact issues. In the spirit of the Heidelberg Appeal [122], it may be advisable to start the discussion of the impact of antimicrobial agents by placing the arguments concerning the risks in some form of sociological and psychological context.

Ames [123] has identified a tendency in modern society to espouse an apocalyptic view of our planet's future and to base fears of the threat posed by pollution on weak or bad science. This problem is not confined to non-scientists. There are clearly identifiable forces operating in science that may lead to an over emphasis on, or an exaggeration of, the potential risks of any phenomenon. One might be termed 'grant application bias'. Research is totally dependent on the availability of adequate funds. When applying for grants to fund research, it is common, if not obligatory, to suggest that the phenomenon one proposes to study is real, of vital importance, and requires urgent investigation. There is an understandable temptation not to minimise the potential risks the phenomenon poses to the environment or to human health. It is not uncommon that scientists are obliged to insist on a significant risk associated with a phenomenon, in order to obtain the money necessary to investigate the existence and magnitude of that risk. Frequently, the development of the technical skills needed to investigate a phenomenon, involve the whole of a research group's mental and physical activity. There is no time, or, given the increasing specialization of research groups, expertise, to develop a quantitative analysis of the associated environmental risks. Further, if having established a funding and expertise niche, one's investigations indicate that the risks associated with the phenomenon under examination are of little significance; there is a clear temptation not to stress this conclusion. This 'grant application bias' may therefore result in the publication of a disproportionate number of papers that address potential risks to the environment or to human health. It may also result in a number of technically competent papers analysing phenomena in which comments on the associated risks are made, frequently in the discussion section, without the presentation of any data on the

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assessment of such risks.

A second, and related, force towards over emphasis is a consequence of 'publication bias'. Publication bias has been defined as the consequence of the fact that a study demonstrating "statistically significant" results is more readily submitted to a scientific journal and, if submitted, more readily publishable than a study whose findings are not statistically significant [124]. Thus, the operation of this bias results in the selective dissemination of papers that demonstrate correlations. Work that demonstrates a lack of correlation is frequently required to be more extensive than work that demonstrates a correlation. Overall, this bias results in a body of scientific literature that highlights potential risks rather than objectively assessing their importance.

A third force that can be postulated to be operating within science can be termed 'funding source bias'. This may have the effect of reducing the emphasis on the risks associated with a phenomenon. When the phenomenon under suspicion is the result of the actions of a large commercial company, that company may have sufficient resources to fund research investigations in both its own, and outside laboratories. In the field of chemotherapy, it is clear that there are companies that fund research in external laboratories. It would be extremely naive to assume that the source of funding would have no effect on the style and scope of the investigations, or on the data finally published. The increasing frequency, with which 'confidentiality agreements' are being required by such companies, does nothing to allay these fears. The fact that, in addition to funding research, these multinational companies can and do fund conferences and make major contributions to the survival of scientific journals, is a factor that has received too little serious analysis amongst scientists who enjoy the benefits of such patronage.

Scientists like to think of themselves as being open-minded seekers after truth. Lewontin [125] has argued that this attitude takes little cognisance of the philosophical, sociological, political or economic context within which science is performed. At the philosophical level, it has been argued that the linear-causal reductionist paradigm that currently dominates science may not be the most suitable framework for addressing the complex interactions that are encountered in environmental issues [126]. At the psychological level, the well established phenomenon of cognitive dissonance, would suggest that people, and this must also include scientists, have a tendency to interpret any

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new evidence as support for their previously stated positions. Laurence Sterne [127] identified this tendency over two hundred years ago when he wrote, in his novel *Tristram Shandy*, "It is the nature of a hypothesis when once a man has conceived it, that it assimilates everything to itself, as proper nourishment, and from the first moment of your begetting it, it generally grows stronger by everything you see, hear or understand."

It is clear, therefore, that any review of the published literature on the impact of non-human use of antimicrobial agents must be predicated on the fact that the literature does not necessarily present an objective assessment of the problem. In assessing the risks associated with the impact of the use of these agents in fish farming, a reviewer is faced with a further problem. There exist limited data of specific relevance to fish farming. There are, however, considerably more data available on the risks associated with the use of these agents in land-based veterinary medicine. A review of these data is presented below. The general conclusions, the problems that have been encountered, and the approaches that have been productive in studying this use of antimicrobial agents may well provide a valuable guide for the study of the risks associated with fish farming.

Resistance to antimicrobial agents in human pathogens

The major concern that has been expressed about the use of antimicrobial agents in non-humans is that it may have an adverse impact on the therapy of human disease. The postulated chain of events would involve the selection, in the non-human environment, of a large number of plasmids coding for resistance to multiple antimicrobial agents and the subsequent transfer of these R plasmids to human pathogens with a resultant unlimited spread of resistance determinants in the population of human pathogens [116]. Accordingly, as a preliminary approach, it might be prudent to establish whether there has been an unlimited, epidemic spread of resistance to antimicrobial agents in human pathogens. Even this limited question cannot be answered simply. Wiedemann [128] has argued that despite an enormous number of publications, our knowledge of the epidemiology of antibiotic resistance is still fragmentary. One is haunted by Mark Twain's comment "The researches of many commentators have already thrown much darkness on this subject, and it is probable that, if they continue, we shall soon know nothing at all about it" [1].

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Prior to the use of antimicrobial agents to control infectious diseases the frequency of resistance to these agents in human pathogenic species was probably low [129]. Studies in the early 1970's demonstrated that their use had resulted in a rise in the frequencies of resistant strains and, with respect to some agents and in some species, this rise had been rapid and to high levels [130-132]. Over the last 20 years a number of large-scale, multicentre studies of the frequency of resistance in human pathogens have been conducted to determine the further changes in these frequencies with respect to time. These studies have not provided evidence for the continued, epidemic spread of resistance in human pathogens. O'Brien et al. [133] have suggested that the frequencies of resistance may, in many species, have stabilised at a level characteristic of a particular geographic region. Antibiotic prescribing policies may have played a role in this stabilisation but it is also possible that the overall frequency of resistance in a species may reflect aspects of the ecology of resistance determinants within that species.

In a survey of almost 6,000,000 strains from 242 hospitals in the US the sensitivities of the pathogens isolated during the period 1971-1984 were recorded [56, 134]. With respect to the most pathogens the frequencies of resistance to 16 most commonly used antibiotics remained stable during this 12 year period. In 1983, for example, 140,602 strains *Escherichia coli*, representing 20% of all isolates, were examined and the frequencies of resistance to ampicillin, tetracycline, gentamicin and tobramycin were 31%, 26%, 2% and 3% respectively. The frequency of resistance to ampicillin showed an 8% increase since 1973, the resistance to gentamicin and tobramycin showed no change and the resistance to tetracycline had declined by 2%. In only two of the nineteen species examined, *Staphylococcus epidermidis* and *Streptococcus faecalis*, which represented 13.8% of the strains, examined, was an increase in the overall frequency of resistance recorded. Lorian [135] reported on the sensitivity of *Salmonella* isolated in the US. He noted significant decreases in the frequency of resistance between 1975-1984. The results obtained by Kresken and Weidemann [136] from a multicentre study in central Europe failed to detect any increase in the overall levels of resistance. An international review carried out by the US National Institute of Health published in 1987 [137] confirmed that there had been no increase in resistance to first generation antibiotics in advanced countries. A more recent study of

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86,000 strains isolated in a district general hospital in England between 1984 and 1991 [138] provides no evidence that this situation has changed in the last decade. They reported that the frequencies of resistance had remained relatively unchanged during the survey period. Although O'Brien et al. [133] characterised the frequencies of resistance in the US, in the period 1977 to 1980, as relatively low, the studies have, in general, confirmed that significant frequencies of resistance are encountered in human pathogens. On the basis of the results of these large-scale surveys, Walton [139] has argued that the fears that motivated the Swan report [116] were unfounded and that, in the absence of any evidence of the continued or unlimited epidemic spread of resistance in human pathogens, there was no case for veterinary use of antimicrobial agents to answer.

In contrast to these large-scale surveys that failed to identify significant rises in resistance, numerous recent reviews exist reporting increased resistance as being a major problem in the therapy of infections in hospitals [140-143]. Mayer [144] has suggested that this apparent conflicting evidence is a function of the fact that the increases in resistance frequency in human pathogens tend to occur in specific niches in specific hospitals. Similar, localized outbreaks of bacterial resistance were also noted by Atkinson and Lorian [134]. Thus, although the use of antimicrobial agents in non-humans can not, in recent years, have had a global impact on the frequency of resistance in human pathogens, the possibility remains that it may have had an impact in specific, localised situations.

Mayer [144] identified the specific niches in hospitals where increased resistance was a problem as being those units where patients were immunocompromised, subject to invasive procedures, or obliged to remain for a long time. This would suggest that the selective pressures for the emergence of these resistant strains are internal to the hospital environment. Not only do the problems associated with resistance tend to be confined to certain parts of hospitals they also tend to be greater in large tertiary care and teaching hospitals than in smaller non-teaching hospitals [145]. It is probable that the majority of infections that require treatment in US hospitals are, in fact, acquired in those hospitals. The distribution of species that are isolated and tested for their sensitivity in US hospitals [56] is remarkably similar to the distribution that has been observed in studies of the etiological agents of nosocomial infections [146]. Thus, problems encountered in

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antimicrobial therapy of infections in hospitals are primarily those related to pathogens acquired in those hospitals whose resistance is a function of the antimicrobial agent use in that hospital. Shah et al. [147] analysed the frequency of multi-resistant strains in community-acquired and nosocomial infections and concluded that the data did not support the hypothesis that such strains were selected by veterinary use of the agents. They did suggest that, since there was a 'remote danger' of transferring multi-resistant pathogens from animals to man, continued close monitoring was advisable. Wiedemann [128] has stated that "the use of antibiotics in veterinary medicine probably had an extremely low influence on the problem of antibiotic treatment of human infections in hospitals". In theory, the use of antimicrobial agents in animals could have an impact on the treatment of human disease either by the direct transfer of resistant pathogens from animals to humans, or indirectly by the transfer of R plasmids from animal pathogens to human pathogens. An analysis of the evidence for the existence of either of these transfer processes is presented below.

Transfer of resistant pathogens from animals to humans

Many bacterial species are pathogenic for both animals and humans. McNeill [148] has suggested that the number of microbial pathogens man shares with an animal species is a function of both the length of time and the intimacy of our contact with them. This would suggest that transfer of pathogens between the two host groups is a fairly common phenomenon involving a significant number of pathogenic species. Not all zoonoses are relevant to the argument concerning the transmission of antibiotic-resistant pathogens from animals to man. Only those zoonoses that are treated with antimicrobial therapy in the non-human host can play an active role in this process. Pathogens such as *Yersinia pestis* whose reservoir is in wild animals, or the mycobacteria and the brucella whose infections of farmed animals are not treated with antimicrobial therapy, will therefore, not be discussed.

There are a number of bacterial species that contain both human and animal pathogens and whose infections of farmed animals are treated with antimicrobial agents. However, a bacterial species is a loosely-defined concept and its meaning may vary from species to species even within the same genus [149]. In the *Aeromonas*, for example, A.

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salmonicida is a very homogeneous group [150] whereas, in contrast, the *A. hydrophila* group is extremely heterogeneous [151]. In the present discussion, the concept of clonality allows more precision than that of species. A species may include a number of genetically distinct clones that differ in many properties, including that of host specificity [152]. The significance of this can be illustrated by reference to data on *E. coli* a species whose genetic structure is strongly clonal [153]. Strains of *E. coli* can cause disease in both farm animals and humans. Strains of *E. coli* of animal origin can colonise, at least for short periods, the human intestine [154]. However, there is no evidence to indicate that strains of antibiotic-resistant *E. coli* from animal sources have caused infections in humans [155]. These data suggest that, in *E. coli*, human and animal pathogenicity are properties of separate clones or groups of clones. Similar differences have been noted between the strains of *Staphylococcus aureus* that cause infections in chickens and those that cause infections in humans [147]. There is, on the other hand, evidence suggesting that clones do exist, in some species, that can infect both humans and animals, and that resistant variants of these clones have transferred from animals to humans. This phenomenon has been observed with respect to the *Salmonella* [155-161]. The epidemiology of infections by *Campylobacter* sp. suggests that animal to man transfer is significant [162, 163] and Endtz et al. [163] have presented circumstantial evidence that the frequency of quinolone resistance in human isolates has risen as a result of the use of these agents in poultry production. The significance of these observations is limited by the infrequency with which *Campylobacter* sp. intestinal infections of humans require antimicrobial therapy [164, 165] and the non transferable nature of quinolone resistance determinants [112]. Evidence has also been presented that animal to human transfer of resistant strains has been observed with respect to *Yersinia enterocolitica* [166]. Again human enterocolitis caused by this species is not normally treated with antimicrobial therapy [167]. Tauxe et al [168] have reviewed the data available on human disease caused by the transfer of antibiotic-resistant pathogens from the farm environment. All the epidemiological data presented in this review related to the transfer of *Salmonella* sp. from farms to man. Budiansky [169] and Brunton [170] both raised serious doubts as to the quality of the evidence presented by Holmberg et al. [157] for such transfers. In particular, Budiansky [169] noted that the one patient, who died in the outbreak

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investigated, was not infected by hamburgers, nor from any other animal source, but by the medical profession via an inadequately disinfected sigmoidoscope. This topic was debated in a series of letters to the editor of the journal Nature [169, 171-173]. Despite this disagreement about the quality of the evidence it would be imprudent to ignore the possibility, if not the probability, of animal to man transfer of resistant strains of Salmonella. It should be noted, however, that the existence of clones of Salmonella that are capable of infecting both animal and human hosts is not evidence that this is a general property of all the members of that genus. Plasmid profiles of *Salmonella* isolated from both human and bovine sources would suggest that the two sets of isolates represent different populations (R. Helmut, unpublished results, cited in [128]). Walton [174] has gone as far as to suggest that, with respect to intestinal microflora, "It is highly likely that the physiological barriers present in man, animals and birds prevent the successful colonisation by bacteria from an unrelated species whether the bacteria are antibiotic-resistant or not."

Transfer of R plasmids from animal pathogens to human pathogens

Studies on the evolution and ecology of antibiotic resistance genes strongly suggest that horizontal transfer in the environment, including that mediated by R plasmids, has had a significant role on the distribution of resistance determinants [175-177]. However, intra- and interspecies R plasmid transfer has infrequently been observed in vivo [168]. Under experimental conditions, transfer in the human intestine, of R plasmids from animal strains to resident strains has been demonstrated [178]. The epidemiological evidence of Shoemaker et al. [179] also suggests that R plasmids originating in animal strains can transfer to human strains and that such transfer may have occurred in the human intestine. It is possible that R plasmid transfer was responsible for some of the phenomena that have been analysed as presenting evidence for the transfer of resistant Salmonella from animals to man [155-161]. The quality of the evidence does not allow the separate identification of the contribution of these two mechanisms. Two studies have specifically attempted to establish evidence for R plasmid transfer between animals and humans during their normal contact. Levy et al. [180] demonstrated the transfer of a plasmid from chickens to two workers on a chicken farm. In each case, the

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plasmid was isolated on only one occasion and did not persist in the human host. In a more detailed study Parsonnet and Kass [181] and O'Brien et al. [182] examined the restriction fragments of plasmids originating from strains causing urinary tract infections in women working in a poultry processing plant. These were compared with the fragments from plasmids isolated from the poultry microflora. They detected no evidence for the transfer of plasmids from the microflora of the chickens to the pathogens infecting the women. Kayser [145] has concluded that there are no data demonstrating that R plasmids originating in animals have transferred to human pathogens and subsequently caused difficulty in treatment. The diversity of R plasmids present in both animal and human isolates [182] presents significant logistical problems in detecting such transfers in the natural environment. For this reason, O'Brien et al. [182] have argued that the absence of data can not be taken as evidence of the absence of the phenomenon.

Risks associated with fish farming

Midtvedt and Lingaas [120] have argued that there is a risk to human therapy associated with the use of antimicrobial agents in fish farming. Other authors have also adverted to this risk [33, 183, 184]. In Norway stringent regulations have been proposed, in response to this perceived risk, to control the use of antimicrobial agents in fish farming [185]. The important question is, however, not the existence of a risk, but the magnitude of that risk. All human activities impact on the environment. In all cases, there is a risk that such impacts may, at some level, be deleterious to what is currently perceived as the best interests of the biosphere in general, or humans in particular. In order to minimise these risks, it is necessary that some human activities must be regulated. It would be impossible, however, to produce regulations that would remove all risks associated with human activity. The zero risk option, although seductively attractive to campaigning politicians and environmental pressure groups, is neither realistic nor economically justifiable. However, the risks attached to different human activities are obviously not equal [186, 187]. As all regulation is expensive it is essential that an attempt is made to quantify the risks associated with an activity prior to any decision as to its regulation [188].

In order to assess the degree of risk to human health resulting from the use of

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antimicrobial agents in fish farms, it is useful to consider two sources of risk independently. Firstly, there is the risk associated with the transmission of resistant human pathogens from fish farms to humans. The frequency of resistance in these human pathogens may have increased as a direct result of enrichment by the use of antimicrobials in fish farms or as a result of a mating event with an R plasmid donor in that environment. The second risk is associated with the movement of R plasmid-containing bacteria, which are not human pathogens, to the human environment and the subsequent transfer of these R plasmids to human pathogens.

Risks associated with resistant human pathogens transferred from fish farming.

The arguments presented above concerning risks associated with veterinary use of antimicrobial agents suggest that the significance of the transfer of resistant human pathogens from fish farms and their immediate environment to human hosts should be addressed first. The extent to which fish can, and do, act as vectors of human disease is an important parameter to be quantified. In attempting this quantification one can either look at the data on fish as vectors of human disease or look at the public health significance of the pathogens that have been identified in fish or their immediate environment.

Inglis et al. [189] have recently reviewed fish as vectors of disease and illustrate the difficulties that would be encountered using the available data. The data, where available, frequently groups cases caused by intoxications, with a non-infectious aetiology, together with those caused by infectious agents including viruses. In addition it rarely differentiates between cases where shellfish rather than fin fish are the vector or between infections caused by organisms that derive from fish and their environment, from pollution of the marine environment, or become associated with fish during processing, marketing and preparation of the fish. These factors make it impossible to assess, from these data, the extent of the risk associated with the transfer of antibiotic resistant bacteria from fish farms and their environment to humans. Inglis et al. [189] have, however, concluded that the frequency of fish as vectors of human disease is relatively limited. Its importance, however, shows wide variation between countries depending primarily on variations in climatic and cultural factors. As a general rule, the

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importance of fish as a vector is higher in warmer countries and in those with a tradition of raw fish consumption. It should always be borne in mind that, in many cases where a bacterial disease is transferred from fish to man, the outcome is a self-limiting gastroenteritis. Such infections are not treated with antimicrobial therapy [164]. Consequently, resistance determinants in the etiological agents are of no significance.

An alternative approach is to determine the extents of morbidity and mortality associated with the bacteria that have been isolated from fish and their immediate environment. In developing their arguments on the risks associated with therapy in fish farming, Midtvedt and Lingaas [120] cite Austin and Austin [190] as suggesting that there are approximately 20 groups of 'microbes', pathogenic for humans that have been isolated from fish. With respect to the risks to human therapies from antimicrobial agent use in fish farming, reference to this list is misleading. Of the 20 groups of potential pathogens, many are eukaryotes or viruses and as such they can have no relevance to problems associated with antimicrobial resistance. The bacterial species listed by Austin and Austin [190] are shown in Table 3. *Clostridium botulinum*, which was included on their list, is not an infectious agent and intoxications caused by this organism are not treated with antibiotics. Thus, this organism has no relevance to the discussion here and has not been included Table 3. The remaining organisms have been grouped according to their dominant route of entry into the host. Those organisms that gain entry via the mouth may be expected to have, via food consumption, a greater potential to gain access to new hosts than those where the route of entry is primarily the skin. This second group will require physical contact with the host and, as such, any infections will tend to be limited to workers in the industry rather than the general public. With respect to *A. hydrophila* the intestine is thought to be the origin of most septicaemic infections although skin contact can lead to wound infections [191].

Table 3. Bacteria of significance as human pathogens isolated from fish or their immediate environment and the preferred antimicrobial agents for the treatment of infections they cause.

Pathogen ^a	Disease	Preferred treatment ^b
Pathogens primarily entering the host via the mouth		
<i>Salmonella spp</i>	Food poisoning	Ampicillin, amoxicillin, trimethoprim-sulphamethoxazole
<i>Vibrio parahaemolyticus</i>	Food poisoning	No mention
<i>Campylobacter jejuni</i>	Gastroenteritis	Erythromycin
<i>Aeromonas hydrophila</i>	Diarrhoea	Ciprofloxacin, norfloxacin, trimethoprim-sulphamethoxazole
<i>Aeromonas hydrophila</i>	Septicaemia	Cephalosporins
<i>Plesiomonas shigelloides</i>	Gastroenteritis	Trimethoprim-sulphamethoxazole, tetracycline, ciprofloxacin
<i>Edwardsiella tarda</i>	Diarrhoea	Ampicillin
Pathogens primarily entering the host via the skin		
<i>Pseudomonas aeruginosa</i>	Wound infection	Aminoglycoside ± antipseudomonad penicillin
<i>Pseudomonas fluorescens</i>	Wound infection	No mention
<i>Mycobacterium fortuitum</i>	Mycobacteriosis	Amikacin+ ceftoxitin
<i>Mycobacterium marinum</i>	Mycobacteriosis	Rifampicin+ethambutol, trimethoprim-sulphamethoxazole
<i>Erysipelothrix rhusiopathiae</i>	Erysipeloid	Penicillin
<i>Leptospira interrogans</i>	Leptospirosis	Penicillin G. ampicillin

^a after Austin & Austin [190]. ^b after Bartlet [167].

Table 3 presents a useful starting point when attempting to quantify the size of the risk that antimicrobial therapy in aquaculture could present, via the transfer from the aquaculture environment to man of resistant variants pathogens. It should be noted, before starting this analysis, that the presence of a species in the Table 3 does not imply that it is regularly isolated as part of the normal commensal flora of fish. The list includes some species that have only been isolated from fish infrequently. A more detailed consideration of the bacterial species that have been isolated from fish has been presented by Cahill [192]. For the purposes of this analysis, risk can be defined either in terms of increases in overall population morbidity and/or of the numbers of premature human deaths. Major factors to be considered when assessing the extent of such risks are the overall frequency of these infections in humans and the extent of the morbidity and mortality that result from them and the relative significance of farmed fish and their immediate environment as vectors of the diseases. Finally, but importantly, it is also necessary to determine the extent to which the diseases, in humans, are treated with antimicrobial therapy.

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Intestinal infections

A very large percentage of intestinal infections in humans result in disease conditions which are not of sufficient severity to require medical intervention. For this reason, precise data on the morbidity resulting from such infections are practically impossible to obtain [165]. However, it is generally accepted that diseases caused by the first three bacteria in Table 3 namely, *Salmonella* spp., *Vibrio parahaemolyticus* and *Campylobacter jejuni*, are significant agents of human morbidity [165]. *A. hydrophila*, *Plesiomonas shigelloides* and *Edwardsiella tarda* are of slight, if any, importance as enteric pathogens. Holmberg [193] has stated that evidence to indicate that *A. hydrophila* is an enteric pathogen of immunocompetent hosts, is patchy and confusing and that the status of *P. shigelloides* as an enteropathogen is, at best, controversial. The enteropathogenic status of *E. tarda* is unproven [194] and the best circumstantial evidence for its involvement comes from a study of the Orang Asli, a jungle tribe from West Malaysia [195]. Thus, the extent of risks associated with resistant variants of these three putative pathogens is not worthy of further consideration.

With respect to vector systems, fish, particularly raw or undercooked fish, probably play a dominant role in the transmission of *V. parahaemolyticus*. However, it should be noted that 96% of *V. parahaemolyticus* strains causing gastro-enteritis in humans are Kanagawa-positive whereas this property is detected in only 1% of isolates from sea fish [196]. It is possible therefore, that the majority of strains of this organism associated with fish farms are not pathogenic for man. With respect to *Salmonella* spp. and *C. jejuni* infections, contaminated food products and infected humans are the dominant vectors of transmission. A wide variety of food products have been identified as being vectors of these diseases, but fish, from any source have rarely been implicated [162, 197]. *Salmonella* spp. are the group for which the evidence for transfer of resistant strains from animals to man is strongest. These bacteria are capable of multiplying in land based animals and are, therefore, actively growing when they are exposed to antimicrobial agents in such animals. Resistant *Salmonella* have been isolated from samples of fish [198]. Most (125/135) were isolated from molluscs and crustaceans. However, there is no evidence to show that *Salmonella* spp. or for that matter *C. jejuni*,

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can multiply within the aquatic environment or that of fish farms [199]. Thus, even in the rare instances where fish may have played a role in transmission of resistant *Salmonella*, this role can be only as a passive component of human-human or animal-human transmissions.

Antibiotic therapy of intestinal infections by the bacteria in Table 3 is rarely beneficial and is not recommended [164, 165]. Thus, although infections by *Salmonella spp.*, *C. jejuni* and *V. parahaemolyticus* may cause significant morbidity, the extent of the morbidity will not be influenced by any increase in the frequency of antibiotic resistance in these strains. Antibiotic therapy is occasionally indicated in infections caused by *Salmonella spp.* or *C. jejuni* during which serious complications develop [161, 165]. The frequency of resistance can, therefore, have an impact only via therapeutic failure in these small percentage of life-threatening infections. The maximal size of such an effect can, therefore, be deduced from the data on mortality caused by these infections. Blaser [162] has noted that the mortality resulting from diarrhoeal diseases in the developed world is low and is generally confined to the very young and the very old. In the US, the average annual deaths, during the early 1980's, caused by *Salmonella* infections from all sources, were 95 [200]. This figure can be compared to the annual toll of 7,000 deaths that result from falls in the home in the US [187]. These deaths also occur most frequently in the very old. In Ireland, during the 1980's, the average number of deaths each year from all causes was just over 30,000. Of these an average of 1.6 died as a result of *Salmonella* infections and an average of 10 due to intestinal infections of any sort. From this data, it is difficult to postulate any impact of resistant pathogens derived from aquaculture on deaths due to intestinal infections in Ireland.

Other infections

Erysipelothrix rhusiopathiae, *A. hydrophila*, *Leptospira interrogans* and *Pseudomonas spp.* are widely disseminated in nature, and there is no evidence that their distribution is in any way affected by the existence of fish farms [192, 201, 202]. Wound infections by *Pseudomonas spp.*, which can cause serious problems in hospitalised patients whose defence mechanisms are impaired, are normally acquired from sources within the hospital environment. Infections by these organisms,

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acquired in the community, can result from contact with a wide variety of animate and inanimate sources including the patient himself [203]. *E. rhusiopathiae* is a serious pathogen of swine. Infections of humans occur through skin lesions, following contact with faecally-contaminated water. Infections are largely limited to veterinarians, packing-plant workers and others who handle animal products. The resultant infections are frequently self-limiting and are rarely fatal, except in those who consume excessive amounts of alcohol [204, 205]. *L. interrogans* can cause disease in a variety of wild and domestic animals and infections of man normally occur following contact with, or ingestion of, water or soil contaminated by the urine of infected animals [206]. Most human infections are self-limiting although a more severe form, Weil's disease, can occur. In this condition, antimicrobial therapy is of value only in the first 4 days following infection [206, 207]. From the above considerations, it is clear that infections by these four agents present a greater potential risk to workers within the fish farming industry, than to the general public consuming the product of these farms. A survey of the health status of workers in the industry might provide some information regarding the extent of the risks to which they are, in fact, exposed. With respect to the general public, it is unlikely that the cessation of all fish farming would have any impact on the overall incidence of these infections.

Mycobacterium marinum is quite widely distributed in nature and has been isolated from freshwater and marine fish [208]. Swimming pools and tropical fish aquaria have been specifically implicated in infections by this pathogen [209]. Infections by this organism are confined to the colder surfaces of the body and are normally self-limiting [208]. *Mycobacterium fortuitum* is also widely distributed and has also been isolated from aquarium fish. The organism has been associated with injection abscesses [208]. Serious infections have been reported following open-heart surgery, venous stripping and in renal homograft recipients [209]. The antimicrobial agents recommended for use, in those infections by these mycobacteria where therapy is required, do not include those agents in regular use in fish farming [210] (Table 3).

The quinolone group of antibiotics is often used to treat fish diseases and there are data to show that significant levels of resistance to them has developed in fish farms and their immediate environment (Tables 1 and 2). Midtevd and Lingas [120] have argued

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that their use in fish farming represents a serious risk to human health and should be banned. Resistance to the quinolones has, however, been reported not to be plasmid-encoded [112]. Any risk associated with the use of this group of agents must, therefore, be a function of the following three processes; the enrichment of quinolone resistant human pathogens in the fish farm environment; the transmission of these resistant human pathogens from the environment of fish farms to humans and the development of infections in humans which, as a consequence of their quinolone resistance, cannot be treated. The arguments outlined above do not support the hypothesis that farmed fish represent an important vector system for diseases that result in significant human morbidity or mortality. In those hypothetical cases where farmed fish are a vector of any of these diseases, the importance of quinolone resistance in the pathogens is dependent on the agents, if any, that would be chosen to treat the diseases. Of the list of pathogens in Table 3, the quinolones are listed as the preferred treatment only for intestinal infections by *A. hydrophila* and *P. shigelloides*. These infections are rare and antimicrobial therapy is not normally recommended [165, 167].

Risk associated with R plasmids derived from fish farms or their environment.

In order to discuss the possible significance of the transfer of R plasmids from fish farms to human pathogens, it is useful to use the concept of compartments as suggested by Midtvedt and Lingas [120]. For the purposes of this review three compartments, namely the human compartment, the terrestrial veterinary compartment, and the aquatic compartment, will be considered. Midtvedt and Lingas [120] have argued that these three compartments have such a level of exchange of resistances to antimicrobial agents that they must, essentially, be considered as being sub-compartments of a single system. In contrast, the argument presented here is that the three compartment model more accurately reflects our current understanding of this issue.

Given the fact that there are no positive data to demonstrate that problems in human therapy have been caused by the transfer of R plasmids from the veterinary compartment [145], it is not surprising that there is a similar lack of evidence demonstrating R plasmid transfer from the aquatic compartment. The significance of this lack of evidence is, however, limited by the fact that very little work has even addressed

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the problem. In practically the only investigation of this issue, Aoki [108] reviewed the data on restriction fragment and incompatibility group analysis of R plasmids associated with fish farms. He reported that these showed no similarity to R plasmids isolated from human and domestic animal pathogens. The limited number of strains used in this study limits the significance that can be placed on the lack of similarity detected. O'Brien et al. [182] have stated that the diversity of R plasmids in human pathogens means that any systematic study aimed at detecting positive evidence would require the study of a very large number of strains and would present massive logistical and financial problems.

The lack of positive data on this issue and the practical difficulty of obtaining any, means that the degree of risk can be assessed only by inference from our present, limited, knowledge of R plasmid epidemiology. We can start by observing that it is beyond doubt that resistance genes have travelled widely between unrelated bacteria. Data on the distribution of tetracycline resistance genes in different hosts provides, for example, unambiguous evidence that these genes have transferred between bacteria resident in widely different environments [115, 175, 177, 179, 180]. However, the data suggests that there are some differences in the relative frequency of different tetracycline resistant determinants in different hosts. Class D determinants, which are common in fish pathogens [103], were rarely detected in lactose-fermenting coliforms [211, 212] or in Gram-negative isolates from pigs [213]. Class E tetracycline resistance determinant which was found to dominate in plasmids from mesophilic aeromonads isolated from fish farms [98], has not been reported in plasmids isolated from any other species except *E. coli* in which it has been detected in one isolate [211, 212]. However, the ecology of Tet E determinants is far from clear. Lee et al. [213] have recently reported that chromosomally located Tet E is common in *E. coli* isolated in Taiwan, from pigs that have not been treated with any antimicrobial agents. While these data demonstrate that gene movement has occurred, they do not, however, provide any information regarding the frequency of such movements or the mechanisms by which the genes were moved between species. Although plasmid transfer is thought to play a dominant role in gene movement in the aquatic compartment, transposition, transduction and transformation may also have some importance [115, 214]. However, the question being examined here is whether R plasmids, selected in fish farms in the aquatic compartment, can transfer

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resistance genes to human pathogens at a sufficient frequency to present a risk to human therapy. Ervik et al. [183] have recently argued that the available data provides reasonable evidence to suggest that the risk is significant. Essentially their argument suggests that there is ample evidence to indicate that the use of antimicrobial agents in fish farms has selected for R plasmids [33, 105-108, 111]. In this context it is worth noting that R plasmids have been reported to lose resistance genes under low nutrient conditions [215, 216]. Thus, the R plasmids that have been detected in the immediate vicinity of fish farms may not be stable in the adjacent aquatic environment. Ervik et al. [183] further argue that not only have laboratory experiments demonstrated that these R plasmids can transfer at reasonably high frequencies to human pathogens [33, 217], but that it has also been shown that transfer can, and does, occur in both simulations of the marine environment [218-220], the marine environment [214], in the sediments under fish cages [221] and in fresh water farms [111]. Although they admit that significant transfers may occur only at low frequency in the aquatic environment they argue that this low frequency is compensated for by the size of this environment, which allows the experiment to be repeated an almost infinite number of times.

These data primarily concern events that occur in the aquatic compartment. They clearly demonstrate that a reservoir of R plasmids with the ability to transfer to human pathogens can develop in the vicinity of fish farms. The risks to human health associated with antimicrobial agent use in fish farms are, necessarily, a function of events that occur in the human compartment as well as those that occur in the aquatic compartment. It is argued in this review that the events occurring in the human compartment may have the greater influence on the extent of any risk to human health.

In order to clarify the discussion, it must be remembered that in those situations where an R plasmid transfers to a human pathogen in the environment of the fish farm, then the risk is a function of the transfer, with a fish-associated vector, of a resistant human pathogen to a human host. This issue has been dealt with in the preceding section. The scenario under discussion here concerns the situation in which an organism from the aquatic environment of fish farms, which is not itself a human pathogen, is transferred to the human environment together with its associated R plasmid. To present a risk, the R plasmid must then be transferred to a human pathogen in the human compartment, and

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result in a limitation of the therapeutic options in the treatment of any disease subsequently caused by that pathogen.

As discussed earlier, the evidence suggests that the dominant factor determining the overall frequency of R plasmids in human pathogens is the use of antimicrobial agents in the human compartment [128, 222]. The issue to be discussed here is, therefore, the origin of the R plasmids in human pathogens that are available for enrichment by antimicrobial agent use in the human compartment. Theoretically, these R plasmids could have originated in any environment where antimicrobial agents are present. However, for the purposes of this review, the relative contributions from the human compartment, the terrestrial veterinary compartment, and the aquatic compartment, will be considered. These considerations have not been relevant to those studies whose aim has been to establish the risks associated with the movement of recombinant genes from genetically modified organisms [178, 218]. In these cases it must be assumed that the gene in question is unique and therefore represents the only sequence available for enrichment. In contrast, antibiotic resistant genes are widely distributed in all compartments of the environment.

It is important to observe that, in public health terms, small differences in the population of R plasmids present in the human compartment, prior to antimicrobial agent use, will have little impact on the numbers present after the enrichment consequent to this use. It is worthwhile to attempt an estimate of the relative contribution of R plasmids derived from the human compartment, the terrestrial veterinary compartment and the aquatic compartment to the pool of R plasmids available for enrichment by therapy in the human compartment. The two factors that might be expected to determine this relative frequency will be; the relative number of potential donor organisms from each compartment that are present in the human compartment; and the relative stability of any human pathogen-plasmid complexes formed as a result of mating with donors from the three environments. There is, once again, little experimental data that provide information that can be used to quantify these two factors. Therefore the problems can, at present, only be addressed indirectly, or in terms of theoretical probabilities.

With respect to the relative numbers of potential donors, a priori considerations would suggest that there will be more organisms in the human compartment that derive

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from that environment than those originating from the other two compartments. This assumption is supported by evidence that many organisms deriving from the veterinary compartment have a limited ability to persist in humans [174, 223, 224]. If this assumption is correct, then the only way in which this could not be true of the sub-set of organisms containing R plasmids, would be if there was a very significant difference in the frequencies of R plasmid carriage in the microflora of the three compartments. There is no compelling evidence for such a difference. It is therefore reasonable to assume that, cells capable of acting as R plasmid donors, which derive from the aquatic compartment, comprise only a small fraction of the potential donors in the human compartment.

The degree of stability of exconjugate host-plasmid complexes will have a major impact on the public health significance of any transfers of R plasmids that occur in the human compartment. Stability can be a function of either the stability of the plasmid in the individual cell, or of the stability of the host-plasmid complex in the environment. Some plasmids are unstably inherited following their introduction into new host cells [225]. This instability can be so great that the plasmid can not be maintained, even in laboratory batch culture, in the absence of continuous selection. On the other hand, the instability may be very low and be detectable only in continuous culture experiments. These low levels of instability are frequently thought to result from relatively rare generation of plasmid-free strains that, as a result of not having to expend energy on plasmid maintenance, have a faster growth rate than, and outgrow, their plasmid-containing siblings [226]. Although low levels of instability are difficult to detect in the laboratory, they may be expected to have significant consequences for the persistence, over time, of the resistant hosts in the environment. There are, at present, no data to suggest that R plasmids deriving from the aquatic compartment are more likely to manifest these types of instability in human pathogens than those derived from the human compartment itself. At the present state of our knowledge, therefore, the existence of these types of instability has little importance to the argument being developed here.

The stability of plasmid-host complexes in the environment may be of more relevance. This area has, unfortunately, been the subject of little experimentation. Simonsen [227] has argued that plasmids, whether they are fully conjugative or only mobilizable, are highly unlikely to be simply parasitic. Mathematical predictions,

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supported by some experimental evidence, suggest that persistence of any plasmid-host complexes in an environment necessarily requires that the plasmid confers some selective, survival advantage to the host. The application of this argument to R plasmids suggests that they will fail to survive in an environment, unless they either confer such an advantage on their host, or there is a sufficient concentration of antibacterial agent in the environment to maintain a selective pressure. Bennett and Linton [228] have reviewed evidence that also suggests that plasmids may encode properties that result in a selective advantage, in particular ecological niches, for those strains containing them. Their data concerned the survival of R plasmid-containing strains in humans and calves. It is reasonable to postulate that properties of the Inc H2 plasmids, that were shown to enhance the persistence of their *E. coli* hosts in calf intestines [229], would not also confer enhanced persistence in the aquatic environment.

These arguments would suggest that the R plasmid-host complexes that persist in the aquatic environment, in the absence of specific antimicrobial agent selective pressure, must do so, in part, as a result of survival advantages conferred upon the complex by the R plasmid. Laboratory studies have shown that it is probable that these R plasmids, if and when they enter the human compartment, will be able to transfer to human pathogens [33]. What is not probable is that these R plasmids will have the ability to confer on their new host the properties that would enhance the persistence of the new plasmid-host complex in the human environment. There is a significant probability, therefore, that the persistence of new plasmid-human pathogen complexes, formed by mating events within the human compartment, will depend on the compartment of origin of the donor plasmid-host complexes.

The mechanisms outlined above might provide an explanation for the observed distribution of Inc U and Inc C plasmids. As discussed above, in this review Inc A-C plasmids are classified within Inc C. These are the dominant groups that have been identified in fish-associated isolates [105, 108]. In laboratory experiments Inc U plasmids can transfer to, and are stable in, a wide range of hosts. In environmental isolates, however, they have rarely been isolated in hosts other than the *Aeromonads* [105]. The Inc C plasmids have been reported in *A. hydrophila*, *A. salmonicida*, *V. cholerae*, marine *Vibrio spp* and *Edwardsiella tarda* [105, 108, 230]. Hedges et al. [105] suggested that

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these plasmids may have acquired a role amongst bacteria of diverse lineages in this ecological niche. This role is formally similar to the survival functions postulated by Simonsen [227]. These data suggest that plasmids will not persist for significant periods of time, in environments sufficiently different from that of their natural reservoir. It should, however, be noted that there are data demonstrating that plasmids are not necessarily confined to one host. Closely related plasmids have been detected in *Salmonella* isolated from bovine and human faeces [161] and in *Clostridium perfringens* strains isolated from human and porcine faeces [231].

The frequency of the transfer of resistance genes between compartments may be influenced by factors other than the stability of plasmid-host complexes. The transient occurrence of a plasmid in a 'foreign' environment may, via transposition, still play a role in resistance gene movement between different environments. Hedges et al. [105] have suggested that such transposition events may allow the Inc U plasmids of aeromonads to acquire resistance genes during their transient residence in the human compartment. Hummel et al. [232] showed that a resistance gene could spread between pigs and humans independently of the plasmid on which it was originally located. In these experiments the specific gene was located on plasmids of 11 different incompatibility groups. These data suggest that transposition may play an important, and so far little investigated role, in the dissemination of resistance genes.

Conclusions

The question of whether the use of antimicrobial agents in fish farming can have an impact on the success of the therapy of human infections requires a quantitative answer. The arguments and data presented in this review are not adequate to provide such an answer. The investigation, by the US Institute of Medicine, of the risks associated with the use of sub-therapeutic levels of antimicrobial agents in animal feeds provides an example of the type of data required for such an exercise [200]. Even if a quantitative risk assessment, carried out to the satisfaction of experts in the field, were to conclude that the risk was minimal, this, in itself, may not be adequate to allay all fears. It has been clearly demonstrated that the actual extent of a risk is only one of the factors that influences risk perception by the general public [187, 233].

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The continued monitoring of the impacts of the use of antimicrobial agents in aquaculture is essential if we are to adequately address the real and perceived risks associated with this activity. At present, the nature and quantity of the available data precludes the possibility of making a quantitative estimate of the degree of risk with absolute certainty. However a series of arguments have been presented here that suggest that any putative risk to human health is, in all probability, very low. The main elements of these arguments are;

1. There is no evidence of a continuing increase in the frequency of resistance in most human pathogens in the developed world.
2. The level of resistance in these pathogens is primarily a function of use of antimicrobial agents by the medical profession.
3. With the exception of the case of the *Salmonella* there is little compelling evidence that the use of antimicrobial agents, in veterinary medicine, has had an adverse effect on the therapy of human pathogens.
4. Epidemiological and ecological consideration of the pathogenic bacteria that have been associated with fish, suggest that resistant variants of these organisms are unlikely to cause any impact on human morbidity or mortality.
5. Although the frequency of R plasmids is elevated in the fish farm environment, there are reasons to believe that such plasmids make only a very small, and transient, contribution to the numbers of R plasmids in human pathogens.

The data that has been used to arrive at this provisional assessment has, in the main, been derived from studies in developed countries with temperate climates. Additional data would be required to assess the potential risks associated with fish farms operating in developing countries with tropical climates.

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**ASSESSMENT OF THE IMPACT OF ANTIMICROBIAL AGENTS USED IN
THE FISH FARMS ON THEIR ENVIRONMENT.**

You know, this applied science is just as interesting as pure science, and what's more it's a damn sight more difficult.

William Bate Hardy [1]

Two approaches have been made to the assessment of the environmental impact of antimicrobial agent use in fish farms. Each has presented major difficulties both in experimental method and data interpretation. One approach has been to attempt to determine the concentrations of the agents in various components of the environment. The central problem with this approach is that the biological activity of antimicrobial agents is modified, to a significant but variable extent, by the chemical and physical nature of the environment within which they interact with microorganisms [6-8, 234-236]. The second has been to assess the extent of the impact of the agents on processes mediated by bacteria in the environment, or on parameters such as the quantitative or qualitative nature of the microflora, or on the relative size of its resistant sub-population. As a consequence of the problems encountered in *in situ* studies, attempts have also been made to study these phenomena in laboratory simulations [237-242].

Factors that affect the measurement of antimicrobial agent concentrations in the environment.

The majority of quantitative assessments of antimicrobial agent concentrations in fish farms and their immediate environment have been performed using HPLC methods [238, 243-246]. Integral to these methods is an extraction step, which is performed to remove the agent from its association with the chemical and physical factors in the environment, and to concentrate it in an environment where its detection is optimal. The use of a highly efficient extraction protocol is a central feature of a 'good' HPLC method. Thus, a 'good' HPLC method is one that circumvents any interactions between the environment and the agent being assayed. The concentrations of Mg^{++} and Ca^{++} found in sea-water have been shown to reduce significantly the biological activity of oxytetracycline [6] and flumequine [7, 8]. The effect of divalent cations alone may have

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the consequence that the use of a 'good' HPLC method to analyse marine samples will result in the detection of concentrations that overestimate the bioactive concentrations of these agents by a factor of 10-100. These considerations might suggest that the potential of bioassay methods should be investigated. Such assays might be expected to generate data that was necessarily of greater biological relevance. Some authors have published data on the use of plate diffusion assays to detect biologically active antimicrobial agents in marine sediments and in fish intestines [183, 238]. However, the methods used have generated only qualitative data. The selection and design of quantitative bioassay methods has been the subject of little investigation. Any quantitative bioassay would, unavoidably, have the disadvantage that it would not be capable of identifying the chemical nature of the agent mediating any inhibitory effect detected. This specificity is a central advantage of HPLC and other chemical methods. It is also probable that bioassay methods will never achieve the sensitivity that can be obtained by chemical analytical methods. A possible way of producing data that had the necessary sensitivity, specificity and relevance would be to determine the relationship between chemical concentrations and biological activity for any particular environment. By analogy with the term bioavailability, it is suggested that this relationship could be termed bioactivity. The bioactivity in any specific environment could then be used to establish estimates of the biologically-active concentrations from data generated by chemical analytical techniques.

Establishing the dominant parameters that govern the bioactivity of an agent will not be simple. Unpublished work in Galway suggests that the bioactivity determined from in vitro studies will depend on the method used to determine the biological activity. Although all methods used detected significant inactivation, the degree of inactivation was greatest when tube dilution MBC methods were used but was less when tube dilution MIC were used. Plate diffusion assays indicated the lowest extent of inactivation, presumably because they allow the separation of the agent from non-diffusible components of the environment. The extent of inactivation detected by these bioassay methods was also influenced by the choice of indicator organism. The use of strains of different species as indicator organisms, as well as the use of strains of the same species with different resistance levels, has been shown to influence the results obtained [8, 247]. In addition to presenting problems for experimental design, this suggests, not

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surprisingly, that some of the influence of the environment on antimicrobial agent action may be mediated via alterations of the phenotype of microorganisms. Similar environmental effects on bacterial phenotype resulting in changes in sensitivity have been discussed by Brown [38]. The ambiguities of the meaning of the results of laboratory experiments, introduced as a result of the choice of method and indicator organism, might suggest that bioactivity would be better assessed in experiments that employ closer simulations of the natural environment. Hansen et al. [238] have shown that the influence of antibacterial agents on the total numbers, metabolic activity and the frequency of resistance in natural populations can be studied in mesocosms. Attempts to quantify the bioactivity of antimicrobial agents by assessing their ability to select for increased resistance in natural microflora in laboratory microcosms have been made in Galway. This approach has been hindered by the unexpected, and so far unexplained, emergence of high frequencies of resistance to oxytetracycline in microcosms to which no oxytetracycline had been added (unpublished results). Quantitative assessments of bioactivity via the impact of antimicrobial agents on metabolic processes in microcosms have also been attempted. In these experiments, gas production has been used to monitor metabolic activity. In aerobic systems, modifications of methods developed for the detection of antimicrobial agents in fish and other foods have been employed [248]. In these, CO₂ evolution, as measured by conductivity changes, has been used as an indicator of metabolic activity. In anaerobic microcosms, total gas production has been assessed [249]. A delay in the initiation of metabolic activity in marine sediments resulting from the introduction of 6.25 µg g⁻¹ oxytetracycline has been detected in these microcosms.

The problem of environmental modulation of bioactivity is further complicated by the extent to which the impacts are reversible. Oxytetracycline is known to be reversibly bound to clay and other particles [234, 235]. Unpublished work in Galway has demonstrated that oxytetracycline has a very low bioactivity in the presence of some river sediments. The addition of 12% washed river sediment to tube dilution assays increased the MBC of oxytetracycline against *Yersinia ruckeri* from 18 to 1200 µg ml⁻¹ [247]. Soulides et al. [236] have also demonstrated that the biological activity of oxytetracycline is inactivated by binding to clay. This inactivation was shown to be reversible. The inactivation of oxytetracycline and the quinolones by divalent cations in

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sea water is also reversible. The significance of reversible inactivation, in the environment of fish farms, requires very careful consideration. It is easy to understand that, for example, in a river sediment, oxytetracycline may move from the sediment to the water column with a resultant increase in bioactivity. On the other hand, although the inactivation of antimicrobial agents by divalent cations is also reversible in the laboratory, it is not easy to postulate a scenario in which this inactivation will actually be relieved in the environment of marine fish farms.

Thus, it is clear that although HPLC assays are able to produce very misleading results, their replacement with bioassay methods may not necessarily reduce our confusion as to the true impact of antimicrobial agents in the environment.

Methods for the quantitation of resistance to antimicrobial agents in the fish farm environment.

The direct measurement of the size of the sub-population of the total microflora that manifests increased resistance to an antimicrobial agent is important in itself and is, clearly, an important indicator of the impact of that agent in an environment. All studies, in this area, have been faced with serious methodological difficulties and the interpretation of the data generated presents major problems. Many workers, including the authors of this review, have published data on the 'frequency of resistance' in the environment of fish farms or in laboratory simulations of these environments [29, 183, 238, 246, 249-251]. It could be argued, however, that the total number of resistant microorganisms that can be cultured may be a more relevant parameter.

The central difficulty, whether the 'frequency of resistance' or the number of resistant microorganisms is to be reported, is that the culture methods that are available are fundamentally inefficient [252]. The fraction of the total viable microflora that can be cultured on any medium varies, but is probably well below 1% [253]. Samuelsen et al. [246] for example, estimated that they cultured approximately 0.3% of the total viable microflora of the sediments they studied. A second problem arises from the fact that any two media are liable to allow the growth of a quantitatively and qualitatively different sub-population [254]. The choice of medium may therefore influence the frequency of resistance detected. The nature of the environment sampled may influence the relative

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suitability of various media.

A further set of problems derive from variations in the meaning of the word 'resistance'. As noted above, the meaning of this word is context-dependent. The answer to the question; Resistance to what concentration and in what environment? has consequences for the choice of method. The answer to this question will influence the central issue of the choice of concentration of the antimicrobial agent that should be included in selection media. In theory, three approaches could be used. The concentration could be set by reference to the distribution of levels of resistance present in the undisturbed microflora of the environment being examined. Work in Galway, however, suggests that is no discontinuity in this distribution. The fraction of the microflora that can be cultured from unpolluted samples decreases exponentially when the concentration of oxytetracycline is increased in the growth medium. Alternatively, the selection concentration could be set by reference to a breakpoint concentration that had been established for the agent in fish. This would suggest, with respect to oxytetracycline, that concentrations in the range 2-4 $\mu\text{g ml}^{-1}$ should be employed

. In our experience, a high percentage of the natural microflora of undisturbed sediments would be able to grow at these concentrations. A third approach would be to set the concentration at such a level that it would be reasonable to assume that it would select all cells containing R plasmids.

The choice of the selection concentration will also be influenced by the choice of growth medium. This becomes a particularly significant issue in studies of the marine environment where incorporation of sea water, which contains elevated levels of divalent cations, is frequently recommended in isolation media [6]. Samuelsen et al. [246] have attempted to deal with this problem by formulating TSCA in which citrate is added to counteract the inhibitory effects of the cations present in the 70% seawater also used in the medium. Sandaa et al. [33] have, however, suggested that the incorporation of 25 $\mu\text{g ml}^{-1}$ oxytetracycline in this medium may result in an active concentration of approximately 5 $\mu\text{g ml}^{-1}$.

Table 4 Media and antimicrobial agent concentrations that have been used in the selection of resistant organisms from the aquatic environment.

Ref	Study	Samples	Medium	Agent	Conc µg ml ⁻¹
98	Fresh water fish farm	Sediment, water, fish	McConkey agar	Oxytetracycline	30
				Tetracycline	30
183	Marine fish farm	Mussels, fish	Tryptone soya agar	Oxytetracycline	100
				Oxolinic acid	10
238	Marine microcosm	Sediment	Tryptone soya agar + 70% seawater	Oxytetracycline	25,100, 200
				Oxolinic acid	10
				Flumequine	10
225	Marine fish farm	Sediment	Tryptone soya agar + 70% seawater	Oxytetracycline	25
				Furazolidone	25
256	Freshwater	Treated sewage effluent Lake water and sediments	Caesin peptone starch agar	Ampicillin	100
				Chloramphenicol	30
				Erythromycin	15
				Kanamycin	30
				Oxytetracycline	30
Streptomycin	10				
257	Freshwater	Water and sediments from trout farm	Caesin peptone starch agar	Oxytetracycline	100
249	Marine fish farm	Sediment	Zobell 2216V	Oxytetracycline	25
29	Fresh water fish farm	Sediment, catfish, water	McConkey agar	Oxytetracycline	30
				Tetracycline	30
				Ampicillin	10
				Chloramphenicol	30
				Kanamycin	30
Nitrofurantoin	300				
250	Marine environment	Sediment	Tryptone soya agar + 70% seawater	Oxytetracycline	25
				Furazolidone	25
				Oxolinic acid	10
33	Marine fish farm	Sediment	Tryptone soya agar + 70% seawater + 1% citrate	Oxytetracycline	25
241	Marine mesocosm	Sediment	Tryptone soya agar + 70% seawater	Furazolidone	25
246	Marine fish farm	Sediment	Tryptone soya agar + 70% seawater + 1% citrate	Oxytetracycline	25
251	Fresh water fish farm	Sediment, trout, water	Iron agar	Oxytetracycline	8
				Oxolinic acid	1
258	Marine environment	Sediment	Tryptone glucose yeast agar	Ampicillin	50
				Streptomycin	50
				Kanamycin	50
				Chloramphenicol	30
				Tetracycline	30
259	Marine fish farm	Sediment	Tryptone soya agar + 70% seawater + 1% citrate	Oxytetracycline	25
				Trimethoprim	25
				Sulfadizine	300
				Furazolidone	25
				Streptomycin	25

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In practice, a variety of selection concentrations have been used but rarely has the rationale for their use been presented. Examples of the range of selection media and concentrations that have been used in environmental studies are shown in Table 4. In general, they might be expected to select for the majority of the R plasmid-containing strains. However, it should be noted that Datta, one of the founding mothers of R plasmid ecology, recommended the use of 10 µg ml⁻¹ oxytetracycline for the selection of plasmids encoding resistance to this agent [222, 260]. It is, on the other hand, likely that most of the media that have been employed will have failed to select organisms whose resistance levels are high enough to cause problems in the therapy of fish diseases. When the medium and concentration of selective agent to be used have been chosen it is important that media preparation protocols are established and validated. This is of particular importance with media containing oxytetracycline. The half-life of the biological activity of this agent in agar media at room temperature even in the dark is less than 24 hours (unpublished results).

A further problem that occurs when interpreting data on the frequency of resistance in environmental samples, is that of the limited activity spectrum of some of the antimicrobial agents studied. Oxolinic acid, for example, has little or no activity against anaerobes, non-fermenting Gram-negative rods, and very limited activity against Gram-positive organisms [15]. Thus, apparent increases in the frequency of resistance to this agent may result from a decrease in the oxygen tension of an environment with a consequent increase in the frequency of anaerobes. Equally, changes in the quality of the environment that alters the ratio of Gram-negative to Gram-positive organisms in the microflora may also manifest as an increase in oxolinic acid resistance. Similar problems can be encountered with ampicillin and amoxycillin. A significant number of species has been reported to be innately resistant to these agents. These species include the mesophilic aeromonads [230], *Yersinia*, *V. parahaemolyticus* and many other Gram-negative species that are common in fresh water [15]. The ability of yeasts to grow under many of the conditions used to determine resistance frequency has rarely been addressed. In some environments, these non-prokaryotic organisms have represented up to 25% of the oxytetracycline-resistant microflora of sediments [261].

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The validity of the data generated in any field investigation is critically dependent on the methods of sample collection, transport and storage used in the study. The concentrations of both antimicrobial agents and microflora with elevated resistance frequencies in sediments under farms have shown considerable vertical and horizontal variation [245, 246, 249]. Consequently, in sampling such sediments it is essential that the method used will allow the determination of the exact location and depth at which the material to be analysed was collected. Although antimicrobial agents are stable over long periods at -20°C [242] storage of sediment samples at this temperature significantly reduces the number of microorganisms that can be cultured from samples (unpublished data). Standard protocols for the transport of samples requiring microbiological analysis have yet to be formulated and validated.

Studies of the fate of antimicrobial agents in marine fish farms

Studies published on both the fate and impact of antimicrobial agents used in marine salmonid farms have focused on the concentrations of the antimicrobial agents that can be detected by HPLC, and on the frequency of resistance in the microflora [237, 243-246, 249]. In general, the samples analysed have been the under-cage sediments and the non-target fish and shellfish.

Considerable variations exist in the published data with respect to concentrations of antimicrobial agents that have accumulated in the under cage sediments. With respect to oxolinic acid, Björklund et al. [237] reported the concentrations of oxolinic acid detected in sediment under 5 farms. At two farms no agent was detected and at the other three the concentrations were within the range 0.05 - 0.2 ppm. With respect to oxytetracycline, studies have been reported from Finland [237, 244], Ireland [245, 262] and Norway [243, 246] (Table 5). The Finnish studies established that the range of concentrations under five farms was 0.1 - 4.3 ppm [237, 244]. In Ireland, three treatments on the same farm were studied, and the concentrations ranged from 5.5-10.9 µg g⁻¹ [245, 262]. In one Norwegian study, the concentrations under four farms sampled at various times after the end of therapy, ranged from 0 - 4.9 mg kg⁻¹ dry weight [243]. Despite the use of different methods of sampling and analysis, differences in the environment of the farms and husbandry styles, and even the different units in which the concentrations are

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expressed, the results of these studies appear to be in general agreement. However, a second Norwegian study reported a very different picture. Concentrations under two lightly-stocked cages that received oxytetracycline therapy were 285 and 189 ppm [246]. Coyne et al. [262] have considered the factors that might have lead to the large discrepancy between the concentrations they detected in Ireland and those reported by Samuelsen et al. [246]. Both the sampling and analytical methods used in these surveys were similar and neither the area nor the depth of sediment over which the oxytetracycline was dispersed provide any obvious reasons for the differences. The amounts of oxytetracycline used in the farms studied also failed to provide an explanation, especially since approximately 10 times more was used per 15 m cage in the Irish studies (Table 5). The most significant difference between the Irish farm and that studied by Samuelsen et al. [246] was the condition of the under cage sediment. At the Norwegian farm, the sediment was anoxic, black and composed mainly of piles of undigested feed that were, in some locations, 50 cm in height. In contrast, at the Irish farm, individual feed pellets were visible on the sediment surface and the sediment was a hypoxic grey shell sand that supported a significant population of polychaete worms. These observations suggest that overfeeding, or at least the deposition of excess feed on the sediment, may provide one explanation for both the sediment conditions and the very high concentrations of oxytetracycline detected in this second Norwegian study. However, it should be noted that the data of Björklund et al. [238, 245] (Table 5) show that there is no simple correlation between the extent of accumulation of undigested feed under cages and either the concentration of antimicrobial agents in the sediments or their half-lives.

Table 5. The fate of oxytetracycline in sediments under marine fish farms. Comparison of half-lives and concentrations at the end of therapy with inputs and environmental conditions.

OTC input (g/cage/day)	Treatment (days)	Sediment OTC (mg/kg)	Half-life (days)	Current speed (m/s)	Sediment condition ^a	Ref
110	10	285	142	96% < 0.05	Anoxic (50)	246
86	10	189	89	96% < 0.05	Anoxic (50)	246
0	0	26	125	96% < 0.05	Anoxic (50)	246
694 ^b	12	10.9	13	52% < 0.05	Hypoxic (0)	245
865	10	9.9	16	52% < 0.05	Hypoxic (0)	245
1370	24	5.5	>100	52% < 0.05	Anoxic (1-5)	262
530	10	4.3	nr	nr	nr	237
260	10	2.4	nr	nr	nr	237
260	10	2.0	nr	nr	nr	237
200	10	2.0	419	Slack	Anoxic (30)	244
100	10	0.1	9	Moderate	Anoxic (10)	244

nr = not reported; ^a depth in centimetres of anoxic undigested feed layer; ^b Average value from 21 cages.

Oxytetracycline, oxolinic acid and flumequine are not degraded in sediments [242] although they are subject to photodegradation in the upper levels of the water column [263]. Thus, the half-lives of these agents in marine sediments are, in fact, half-lives of persistence and are primarily determined by the rate of resolution [246, 262]. Björklund et al. [237] reported very short half-lives (< 6 days) for oxolinic acid under the cages they studied. The data available on the persistence of oxytetracycline in the sediments under farms show wide variations (Table 5). Björklund et al. [244] reported half-lives of 9 and 419 days and implied that current speed might be an important factor. Samuelsen et al. [246] reported half-lives between 87 and 144 days and suggested that overlaying of oxytetracycline-containing sediments with feed and faeces contributed to prolonged half-lives. This hypothesis was supported by data from aquarium studies [240]. Coyne et al. [245] reported half-lives of 10 and 16 days and suggested that in addition to current speed, bioturbation effected by polychaete worms, that can colonise organically-enriched hypoxic, but not anoxic, sediments played a significant role [264]. In a further study, Coyne et al. [262] observed that over-feeding following therapy resulted in the simultaneous development of an anoxic sediment, disappearance of polychaete worms and a significant increase in the half-life of oxytetracycline in the sediment.

Impact on the frequency of resistance in marine sediments.

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The impact of oxytetracycline use on the frequency of resistance in marine sediment microflora was first investigated by Torsvik et al. [259]. They reported that the frequency under cages remained at between 19 and 38% for up to 13 months after therapy. Samuelsen et al. [246] reported similar long-term effects with frequencies remaining between 10 and 50% for over 500 days after therapy. In this study, resistance frequencies of over 100% were detected in sediment samples 12 days after therapy. Sandaa et al. [33] worked with the same samples but with different aims and analytical methods. Analysis of their data, however, suggests a much lower frequency of resistance in the sediment microflora at this time. The data of Husevåg et al. [255], who detected elevated frequencies of resistance under farms that had closed down up to 19 months earlier, support the concept of long-term elevations of resistance frequencies in Norwegian farms. Studies of the microflora in the sediments under cages in Ireland have revealed a totally different picture [249]. In these studies, there was either no increase in the frequency of resistance, or the increase was relatively low (16%), limited in area, and decreased exponentially with a half-life of 26 days (Table 5). The Irish and Norwegian studies used different selection media and this might have made some contribution to the differences in absolute frequencies detected. However, the differences in method would not have influenced the changes in frequency with respect to time detected at each farm. It is tempting to postulate that the differences in the elevated resistance frequencies and their half-lives reflect differences in both the peak concentration and the persistence of oxytetracycline at the farms (Table 5).

The evidence suggests that the situation may be more complex. None of the available data present convincing evidence of a correlation between antimicrobial agent concentrations in sediments and the frequency of resistance to them in the microflora. Increases in the frequencies of resistance in microflora were detected in marine sediments that were mixed with antimicrobial agents and had been placed, in trays, on the sea floor for 10 and 12 months [250]. Increases in resistant microflora have been detected in laboratory aquaria, after the addition of high concentrations of oxytetracycline (400 ppm), oxolinic acid (100 ppm) and flumequine (100 ppm) [238]. Considering the amounts of the selective agents used, surprisingly low frequencies of resistance were detected in the majority of samples analysed in both these studies. Further, there was, in

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some cases a lack of correlation between the agent added and the agent to which elevated resistance was detected. In the aquaria studies, for example, the highest frequency of resistance to oxytetracycline (50%) was detected following 80 days incubation of a sediment to which oxolinic acid alone had been added. A possible explanation for these phenomena is that strains manifesting MLLR phenotypes [81-83] may be dominant in the resistant strains selected in these experiments. In the data from studies of marine farm sediments, no correlations are evident between the concentrations of oxytetracycline detected by HPLC in sediments and the frequencies of resistance. At the end of therapy Kerry et al. [249] detected elevated frequencies of resistance at sites where no oxytetracycline could be detected. In their studies, Samuelsen et al. [246] detected concentrations of approximately 285 ppm in the sediments on two occasions. However, on one occasion the frequency of resistance was 165%, and on the other, 20%. In this work, the concentration of the oxytetracycline detected in the sediments by HPLC declined exponentially with respect to time but no similar change was detected in the resistance frequencies. This discrepancy between the resistance frequency and the concentration of the putative selective agent is even greater if the data of Hansen et al. [238] on the decline of bioactive oxytetracycline in marine sediment mesocosms is considered. These data show that, in aquaria containing marine sediments, the rate of decline of biologically-active oxytetracycline, but not biologically-active oxolinic acid, is much more rapid than the decline of either chemical as detected by HPLC analysis. Discrepancies between concentrations and resistance frequencies have led Samuelsen et al. [246] to suggest that some of the selection pressure in the sediments they studied, may be exerted by toxic chemicals produced by metabolic processes in the anoxic, organically-rich material under the cages. Kerry et al. [240, 262] have presented data suggesting that some of the resistant microflora detected in sediments at the farm they studied was not the result of selective enrichment in the sediment. Their data suggested that the resistant microflora were deposited on the sediment after their enrichment at an earlier stage and that they may, on at least one occasion, have originated from the microflora present in the feed pellets used at the farm.

Although Lunestad et al. [265] have demonstrated that there is little reduction in the diversity of microbial flora under treated cages; there have been no detailed studies of

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the generic distribution in the resistant microflora under cages in the marine environment. A limited characterization of the microflora in the hypoxic sediments under two farms in Ireland suggests that pseudomonads dominate in the oxytetracycline-resistant sub-population present immediately following treatment [261]. Little detailed information exists regarding the nature of the resistance mechanisms in the sediment microflora. Some, but not all, of these bacteria will possess R plasmids encoding for positive function resistance [33, 108, 189]. However, the existence of other mechanisms of resistance is implied by the data demonstrating the emergence of an increased frequency of resistance to oxytetracycline in the presence of oxolinic acid [183, 238, 250]. Since oxolinic acid resistance is not plasmid-encoded [112] the emergence of these strains manifesting resistance to oxytetracycline cannot be a result of enrichment for R plasmid-containing strains. Thus the underlying resistance mechanism may be similar to that mediating the multiple low-level (MLLR) resistance phenomenon mentioned earlier [81, 82]. To the extent that the increase in oxytetracycline resistance in these strains is mediated by persistence mechanisms, their significance may be limited by their being non-transferable and, in the absence of selection pressure, transient in the environment [44].

No studies published to date have demonstrated a significant correlation between antimicrobial agent concentrations in sediments and the frequency of resistance in the sediment microflora. It is clear, therefore, that the causal factors leading to increases in the frequency of resistance that have been detected in such environments, have yet to be fully elucidated. What is evident, however, is that although the frequency of resistant microflora in a sediment may be important in itself, it can not be used as an indicator of the biological activity of an antimicrobial agent in that environment.

Impact on non-target fish in marine farms

A limited number of studies have investigated the concentrations of antimicrobial agents that can be detected in non-target fish living in the environment of fish farms [183, 237, 244, 266]. Samples of bleak caught near a fish farm in Finland were shown to contain oxytetracycline residues of 0.2 - 1.3 $\mu\text{g g}^{-1}$ following therapy with this agent [244]. No residues of oxolinic acid were detected in bleak following a treatment in which very little of this agent was used [237]. On the other hand, Norwegian studies on eight

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farms have detected oxolinic acid residues in a high percentage of fish caught in the vicinity of fish farms [183, 266]. In general, these data show that the concentrations in wild fish were proportional to the amounts of the agents used on the farms and that the highest concentrations were detected in saithe. At one farm, the mean concentration detected in the saithe muscle was 5.56 ppm. This suggests that these fish were obtaining almost the totality of their diet from the fish farmers [183]. Blue mussels in the vicinity of fish farms have also been shown to contain antimicrobial agents at the end of a period of therapy [266, 267]. The half-life of oxytetracycline in mussels has been shown to be 3-5 days (unpublished results) but the half-lives in fin fish tissues must be expected to be significantly longer [68]. Since the highest concentrations of oxolinic acid, in these studies, were detected in saithe, it is likely that the predominant means by which the agent enters the wild fish is through their consumption of medicated feed pellets [183]. Consequently, these data imply that more pellets were presented to the fish in the cages than they were able to eat. The significance of these Norwegian data to cage culture in general may, therefore, depend on the extent to which the degree of overfeeding in these farms is normal and unavoidable in such culture systems. The hypothesis that there is a greater degree of overfeeding on Norwegian farms compared to Irish farms has been mentioned above. The comment of Enger and Thorsen [268] that outgassing of sediments in the fish farms they have observed is a frequent occurrence might be taken to support this hypothesis. Outgassing is rarely observed in Ireland and, if observed, is treated as being evidence for a serious pollution problem resulting from either poor site selection or poor quality management practices.

Studies on freshwater farms

There is little available data on the fate of antimicrobial agents used in fresh water fish farms. In a study of oxytetracycline use in a salmon hatchery Smith et al. [269] demonstrated that the quasi-totally of the agent left the farm associated with particulate matter that could be removed from the farm effluent by a rotating drum filter fitted with a nominal 40 μm sieve. Increases in the frequency of resistant microflora in farm effluents following therapy have received more attention. None of the published studies provide details of the amounts of therapeutic agents used, the stocking densities, the flow rates or

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effluent treatment systems in the farms investigated [29, 202, 251]. Austin [202] detected increases in resistance frequency in fish farm microflora following treatments with oxolinic acid, oxytetracycline and potentiated sulphonamides. Interestingly, there was little correlation between the therapeutic agents used and the resistances observed in the effluent microflora. The microflora isolated during oxolinic acid therapy, for example, were all (30/30) resistant to ampicillin, chloramphenicol, erythromycin, furazolidone and other agents but only 30% were resistant to oxolinic acid. Since oxolinic acid resistance is not plasmid-encoded [112], selection for R plasmids cannot provide an explanation for this result. Analysis of these data is further complicated by the fact that the increases in the frequencies of these resistances were detected, not only in the outflow, but also in the inflow of the farm. The increases in frequency of resistances detected by Austin [202] were transient, but Spanggaard et al. [251] presented evidence that the use of antimicrobial agents may have long-term effects on the frequencies of resistance in the microflora of farm effluents. MacPhearson et al. [29] presented evidence for increases in single and multiple resistances in isolates from fish farms with a history of antimicrobial agent use. They also reported frequencies of resistance to oxytetracycline, tetracycline and ampicillin in farms, with no recent history of antimicrobial agent use, that were significantly higher than those detected in the microflora of rivers without fish farms. Thus, these data suggest either a long term (over 60 days) effect on resistance, or, as discussed by the authors, that the selection pressure resulting in the increased resistance frequencies may have been mediated by the chemical nature of the feed introduced to the water, or by some other facet of aquacultural management practices. All three studies failed to detect major differences between the species distribution in the microflora of the farms studied and that typical of rivers. The only clear conclusion that can be made from these studies is, that there is highly likely to be an elevated frequency of resistance in the microflora leaving fish farms. There is, in addition, evidence that on occasions, the elevated frequencies persist long after the period of therapy. There have been no quantitative studies on the mechanisms by which these strains acquired increased resistance. Based on the frequency with which R plasmids are encountered in freshwater pathogens [98, 105, 107, 108, 111, 189], it is highly likely that some, at least, of the resistant strains in farm effluents, will harbour R-plasmids.

CONTROL OF THE DEVELOPMENT OF RESISTANCE.

What really makes science grow is new ideas, including false ideas.

Karl Popper [1]

The arguments developed in this review lead to the conclusion that the rate of emergence of clinically-significant resistance to antimicrobial agents in fish pathogens represents the major problem associated with the use of these agents by fish farmers. Schnick [270] and Meyer [271] have argued that the problems associated with the licensing of new antimicrobial agents for aquaculture are such, that a very limited number of new agents will become available in the foreseeable future. The aquaculture industry must therefore face the future without the expectation that the pharmaceutical industry will suddenly produce a solution to the problem of resistant pathogens. Even if new agents are licensed it is probable that their cost would present a further set of problems for the industry. These observations lead to the idea that the use of antimicrobial agents in the fish farming industry is, and will increasingly be, subject to internal negative control. Both the frequency of resistance in pathogens and the increasing cost of therapy will be effective in limiting their use.

Given this scenario, it is important that husbandry techniques that minimise the requirement for antimicrobial therapy are adopted by fish farmers. This issue has been discussed elsewhere [272] and will not be addressed further in this review. It is, however, unlikely that the industry will be able to operate without some level of antimicrobial therapy. Therefore, we must learn to operate fish farms in such a way as to minimise both the emergence and significance of resistant fish pathogens. Aoki [108] has indicated the enormity of this problem and has admitted that he has no idea how to prevent the increase in the frequency of resistance in fish pathogens. The authors of this review share this sense of impotence. The following comments, which are based largely on the recent work on the fate and impact of antimicrobial agents in the environment, present personal suggestions as to the direction that future research in this area might take.

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A slight misreading of the comments seen on a protest banner outside the Academy of Sciences in Baku might provide a starting point. "Without sanitary culture, there would be no culture at all" [1]. Sanitary hygiene has probably had more impact on human and animal health than any other measures, yet the treatment of wastes from fish farming has rarely been addressed from a health perspective. Land-based farms frequently have control of both their water inflow and outflow whereas this is rarely, if ever, the case in open-water cage farms. Thus, in general, land-based fish farms present more scope for the control of the movement of chemicals and microorganisms than are available in open-water cage farms. The data of Smith et al. [269] have demonstrated the potential of outflow filtration to contain oxytetracycline to the internal farm environment. If this is true of other antimicrobial agents, then filtration may have an important role in the reduction of the degree of enrichment of resistant microflora that occurs outside farms.

It is possible that filtration may also play a role in limiting the movement of both resistant bacteria and pathogens into and out of farms. The efficiency of filtration in removing these bacteria will depend on the design of the filter and the extent to which the pathogen is associated with particulate matter. The association of bacteria with particles in the inflow and outflow of fish farms has not been investigated in detail, but a number of arguments would suggest that this might be a fruitful line of research. It is a commonplace of microbial ecology that microorganisms tend to colonise surfaces [273]. Palmateer et al. [274] have shown that, in natural waters, the numbers of bacteria in the water column correlate with its turbidity. Unpublished experiments in Galway have suggested that approximately 80% of the culturable bacteria can be removed from a freshwater farm effluent by a rotating drum filter with a nominal sieve size of 40µm. With respect to pathogens there is every probability that they will be associated with faecal particles leaving fish and will, therefore, demonstrate a higher degree of particle association leaving fish farms than saprophytic bacteria. The extent to which pathogens will be associated with particles in the inflow to the farms will depend on the nature of their reservoir in the wild. For *Yersinia ruckeri* and *A. salmonicida*, wild fish, and possibly the intestines of wild fish [62] have been suggested to represent the main reservoirs in the rivers suggesting that they may, to a large extent, be associated with

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faecal particles when they enter fish farms. In the case of the hydrophobic bacterium *A. salmonicida*, the data of Enger and Thorsen [268] and that of Sakai [275] suggests that even if these bacteria are not associated with fish faeces the degree of particle association may still be significant. The extent to which resistant bacteria, when leaving farms, are particle-associated will depend on the site in the farm where enrichment for such strains occurs. Although data from animals would suggest that oral administration of antimicrobial agents results in significant enrichment of resistant strains in the intestinal flora [276], there is surprisingly little work specifically demonstrating that this is the case in fish. Sugita et al. [277], found no evidence for enrichment of resistant strains in the intestines of goldfish and Björklund et al. [244] reported very little enrichment in the intestines of fish fed oxytetracycline. Austin and Al-Zahrani [278] on the other hand, reported elevated frequencies of resistance in the intestines of rainbow trout following the administration of a number of antimicrobial agents. Ervik et al. [183] detected high frequencies of resistance in the intestinal flora of wild fish in the environment of salmon farms. These data demonstrate that resistant bacteria can be detected in the intestine of fish. Accordingly, it is possible that at least a proportion of the resistant microflora leaving fish farms will be associated with particulate faeces. The results of any experiments aimed at detecting the impact of outflow filtration on the movement of these resistant strains may be of great interest.

The scope for this type of sanitary control in cage farms is considerably less. Systems have, however, been designed to collect the particulate outputs from cage farms [279] and these are in operation in Norway. Initial results of monitoring their impact have demonstrated a significant reduction in the concentrations of antimicrobial agents in wild fish in the vicinity of fish farm cages [280]. The value of such systems may be greater in the situation described in Norway by Samuelsen et al. [246] than that described in Ireland by Coyne et al. [245]. In the Norwegian farm, the quasi-totality of the oxytetracycline used was detected, together with piles of uneaten feed pellets, in the sediments under the cages. In contrast, in the Irish farm, less than 5% of the oxytetracycline was detected in the area of the sediment subject to feed pellet deposition. The majority of the oxytetracycline is assumed to have left the cages at this farm associated with disrupted faecal pellets and to have been moved primarily in a lateral, rather than vertical direction.

CONCLUDING REMARKS

...complex systems can be understood only by meticulous data collection, logical analysis, and repeated practical scale investigations to identify the governing factors in each situation. Neglect of any part of this combination quickly leads to nonsense.

L.P. Smith [281]

This review has attempted to address some of the issues raised by the resistance to antimicrobial agents in microorganisms associated with fish farming. Globally, a large number of fish and shellfish species are farmed using a variety of different husbandry techniques. Fish farms are operated in widely differing climates and cultures. Although many of the issues associated with resistance to antimicrobial agents are common to all fish farming enterprises, each specific situation will present some unique dimensions of the problem. It has been impossible to address all these specific issues in the scope of a single review. Given the need for some limitation in the scope of the review, it was decided to concentrate on the problems of resistance in fin-fish farming in developed countries.

This decision was significantly influenced both by the experience of the authors and by the nature of the published information. Studies concerning resistance to antimicrobial agents in human and veterinary medicine and those concerning the fate and impact of these agents in fish farms have been published more frequently from studies in the developed world than the developing world. Reports concerning these issues as they affect the developing world are so limited and fragmentary that they do not provide sufficient data to justify any attempt, as yet, to construct any overall synoptic statements.

Within this geographical and species limitation, the review has attempted to discuss the potential impact of resistance to antimicrobial agents in three areas. These areas, the operation of fish farms themselves, the therapy of human disease and the health of the environment represent the areas that have been the subject of most research and have generated most comment in recent years [282]. The review has attempted to provide a critical overview of the current state of our knowledge in these areas. With respect to some topics, our knowledge is seriously incomplete and the review has only been able to

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provide an overview of the current state of our ignorance. In these instances, the review has tried to indicate the type of data that is urgently required. With respect to many other topics discussed in the review, the level of our knowledge is insufficient to justify the forming of unambiguous conclusions. This, for example, is true of the discussion of the size of any potential impact on human health. Here, attempts have been made to construct, from the available data, the most probable scenario. Clearly, not all readers will agree with everything in this, or other sections of the review. Such disagreement is the life blood of science. The review has been written to stimulate serious scientific debate. If it has facilitated such a debate by defining the critically important issues that require investigation, then it will have fulfilled its author's ambitions.

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