On the use of citations to scientific literature in "Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment."

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Method of analysis

A detailed examination was performed on a portion of this review that contained 137 citations. The papers referenced in 125 of these citations were read and were classified on the basis of the relevance of the material in the cited paper to the arguments or observations in the sentence or sentences with which the citation was associated in the text of the review.

The sections of the review examined contained approximately half of the words of the review and just over half of the citations. The sections examined were not chosen at random but were selected because they appeared, at first sight, to contain questionable citations.

Summary of analysis

Each of the 125 citations was classified as relevant, marginally relevant, partially relevant, redundant or not relevant. The criteria used in making these judgements are presented in detail in Appendix 1. In addition each cited paper was categorised as a primary or secondary source of data.

The full analysis is detailed in the following page but the Table below provides a summary of the findings.

	Primary source	Secondary source	All
Relevant	7	10	17
Partially relevant	5	3	8
Marginally relevant	-	7	7
Redundant	-	10	10
Not relevant	56	27	83
All	68	57	125

Rationale

Disagreement and debate are the life-blood of science. The more complex and difficult a field and the more indirect the measurement that can be made of key variables, the more one should expect argument and debate.

Listing the potential impacts of antimicrobial agent use in aquaculture is relatively easy. Elucidating the actual impacts and the importance of those impacts is, however, a considerably more difficult and complex task. With respect to these issues disagreement between research workers should not only be expected, it should be welcomed. Disagreement stimulates research and debate. The generation of new and relevant data and the debate about their meaning and significance are the primary mechanisms by which science makes progress.

These notes were not written to explore my disagreements, and they are many, with the positions taken by Cabello in his review. The motivation for preparing these notes was a passionate belief in the absolute necessity of maintaining the integrity of scientific discourse.

If scientific debate is to be productive it is essential that participants adhere to the conventions of scientific communication. One of these conventions requires that, when, in an article or review, arguments or statements of fact are supported by citing the work of other authors, these citations are made with integrity. It is essential that the cited papers actually contain the data or information they are claimed to contain.

In order to confine the discussion to grounds where reasonably objective judgements can be made, these notes have been limited to addressing the relationship between the text of this review and the literature cited in association with that text.

The question of whether a particular paper contains information or data relevant to a particular statement is one that can be established with reasonable objectivity by examining the relationship between the contents of the paper that has been cited and the content of the portion of text for which that paper is cited as providing supporting evidence.

In preparing these notes I have deliberately avoided, wherever possible, making any suggestions as to other sources of data that might, productively, have been consulted and cited.

In the following sections I have attempted to provide a brief summary of the reasons why I have classified 125 citations as relevant, marginally relevant, partially relevant, redundant or not relevant at all.

I have tried to be objective in making these judgements but I am aware that I may have made errors of judgement and may have misread or misunderstood the papers I have read. For this reason, I invite and encourage anybody reading these notes to check for themselves.

Read the review, choose a citation, read it and make your own mind up.

Considerations of the Title of the review

On the use of the word 'heavy'.

The only country for which this review provides a quantitative estimate of the use of antimicrobials in aquaculture is Chile, a country responsible for approximately 2.3% of world aquaculture production. The author, despite making 24 citations to papers (Grave et al, 1996, 1999; Lillehaug et al., 2003; Markestad and Grave, 1997) that provide a quantitative analysis of the dramatic reduction in antimicrobial use in Norwegian aquaculture, has not presented any data on or estimates of the use of antimicrobial agents in any country other than Chile.

I cannot accept that the citation of a single reference, Bravo et al. (2005), is sufficient to justify the use of the word 'heavy' in the Title.

On the use of the word 'prophylactic'.

The word 'prophylactic' to describe antimicrobial administrations in aquaculture occurs in the Title of this review, twice in the Summary and on six other occasions in this text. The author never mentions metaphylactic administrations and appears to classify **all** antimicrobial agent use in aquaculture as prophylactic.

Current thinking would differentiate between therapeutic, metaphylactic and prophylactic use of antimicrobials. A reading of Tabla 1 in Cabello (2003) demonstrates that he is clearly aware of the meanings given to these terms.

The six places where prophylaxis is referred to in the main text are associated with 16 citations of published papers. Some papers are cited in more than one place and in total 9 different papers are cited. Of these, 6 refer to Norwegian salmonid aquaculture, 2 refer to Vietnamese shrimp culture and one (Cabello, 2003) is a review.

Le and Munekage (2004) and Le et al. (2005) represent separate reports of studies at the same Vietnamese sampling sites. One or other must, therefore, be redundant. Neither paper mentions the rationale for the antimicrobial agent use in the shrimp farms investigated and neither use the word 'prophylactic' or 'prophylaxis'.

Of the Norwegian papers, Lillehaug et al. (2003) provides the most detailed treatment of the rationale for therapies that occurred in Norwegian aquaculture. After examining the records of all (5439) administrations of antimicrobials that occurred in this industry between 1991–2000 they classified only one (0.02%) as prophylactic.

Sørum (2006), cited four times, uses the word prophylactic only on one occasion and this is to characterise an administration to an ornamental fish in the US. Grave et al. (1999), which is cited three times, also uses the word 'prophylaxis' once. Here it is used to describe the use of antimicrobials as growth promotors in US land-based agriculture. Both these papers suggest that the majority of antimicrobial use in Norwegian aquaculture was to control furunculosis or, to a lesser extent, vibriosis. This implies that antimicrobial agent use in Norway would, therefore, be more correctly described as metaphylactic.

Neither Grave et al. (1996), Markestad and Grave (1997 or L'Abee-Lund and Sørum (2001) mention prophylaxis or prophylactic treatments.

The use of the word 'prophylactic' in the Title cannot be justified by the references to the papers cited in the text.

Considerations of the use of citations to published literature in this review

Material in ***blue italic bold*** are direct quotes from Cabello's review.

My comments and notes are in black normal type.

Quotes from other sources are in *black italic* type.

Introduction

For example, the aquaculture of shrimp and salmon has been accompanied by an important use of prophylactic antibiotics in the aquatic environment of rivers, lakes and oceans (Grave et al., 1999; Le and Munekage, 2004; Le et al., 2005)

Grave et al. (1999), which addresses antimicrobial use in Norwegian aquaculture, uses the word 'prophylaxis' only once. This use is to describe to describe the use of antimicrobials as growth promoters in US land-based agriculture. This paper implies that the majority of antimicrobial use in Norwegian aquaculture was to control furunculosis. This use, therefore, should be described, more correctly, as metaphylactic.

Le and Munekage (2004) and Le et al. (2005) represent separate reports of studies at the same 4 sampling sites. One or other of these citations must therefore be classified as redundant. Neither paper mentions the rationale for the antimicrobial agent use in the farms investigated nor do they use the word 'prophylactic' or 'prophylaxis'

this use has resulted in an increased antibiotic resistance of bacteria in the environment (Rhodes et al ., 2000a; Miranda and Zemelman, 2002a,b; Petersen et al ., 2002; Alcaide et al ., 2005).

Rhodes et al. (2000a) make no comment on the use of antimicrobial agents in the farm where they collected their samples. The design of their study allowed the demonstration of the presence of resistant bacteria but precluded any comment on any possible increase in either the frequency or numbers of such bacteria.

Miranda and Zemelman (2000a) studied oxytetracycline resistance in farms with "*no history of recent oxytetracycline exposure*". Miranda and Zemelman (2000b) presented a further examination of the strains isolated in Miranda and Zemelman (2000a).

Petersen et al. (2002) studied an integrated farming system where the only antimicrobials used were those administered to the land-based animals and "*None of the fish in the ponds were given any antimicrobial treatment.*"

Alcaide et al. (2005*)* reported *"……., in our study, a correlation between antibiotic usage and antibiotic resistance could not be established, since the sampling events were not correlated with clinical outbreaks and antibiotic therapy"*

The emergence of antibiotic resistance among fish pathogens undermines the effectiveness of the prophylactic use of antibiotics in aquaculture (L'Abee-Lund and Sørum, 2001 Sørum, 2006)

Both L'Abee-Lund and Sørum (2001) and Sørum (2006) discuses resistance to antimicrobials in bacteria associated with fish disease. Neither, however, discusses prophylactic use of antibiotics in aquaculture. Sørum (2006) uses the word prophylactic on only one occasion to describe an administration to an ornamental fish tank in the US.

Antibiotic use in aquaculture

Moreover, hygienic shortcomings in fish raising methods, including increased fish population densities, crowding of farming sites in coastal waters, lack of sanitary barriers and failure to isolate fish farming units with infected animals (Naylor et al ., 2000; Naylor and Burke, 2005), have increased the possibility of rapid spread of infections.

The only relevant, but unreferenced, comment I can find in Naylor et al. (2000) is *"Intensification implies increasing the density of individuals, which requires greater use and management of inputs, greater generation of waste products and increased potential for the spread of pathogens*."

Naylor and Burke (2005), to the extent to which they discuss disease at all, confined their discussion to the transmission of diseases from farmed species to wild species.

They also made the following (unreferenced comments).

" Dense cultures of fish can lead to disease epidemics,"

"Reducing fish stress in net pens and filtering effluent from recirculating tank systems can also help minimize disease transmission."

"In general, large-scale aquaculture provides opportunities for the emergence of an expanding array of diseases: It removes fish from their natural environment; exposes them to pathogens, which they may not naturally encounter; imposes stresses that compromise their ability to contain infection; and provides ideal conditions for the rapid transmission of infectious agents and diseases."

This scenario also results in an augmented use of prophylactic antibiotics, often with the misplaced goal of forestalling these sanitary shortcomings (Grave et al., 1996; Cabello, 2003; Sørum, 2006).

Grave et al. (1996) do not mention the use of prophylactic antibiotics. As they use prescription data to estimate morbidity it is clear that they do not assume the treatments they studied were prophylactic.

Cabello (2003) is a review and cannot be the source of primary data.

I cannot accept that Sørum (2006) makes observations that can be cited in support of the comments made in this sentence

The unconsumed food, and fish faeces, containing antibiotics reach the sediment at the bottom of the raising pens; antibiotics are leached from the food and faeces and diffuse into the sediment and they can be washed by currents to distant sites (Hektoen et al ., 1995; Kerry et al ., 1996; Coyne et al ., 1997; Holten et al ., 1999; Guardabassi et al ., 2000; Sørum, 2000; Sørum and L'Abée-Lund, 2002; Boxall et al ., 2004; Sørum, 2006).

Hektoen et al. (1995) studied the persistence of antimicrobials that had been mixed with sediment and placed in polyethylene boxes on the seabed. Their data have only very limited relevance to the issues of fate and distribution discussed here.

Kerry et al. (1996) did detect oxytetracycline in sediments under a fish farm. However they failed to detect any oxytetracycline in any samples taken over 10 m from the cages. Coyne et al. (1997 investigated oxytetracycline concentrations only in samples taken from immediately under the cages. Neither of these papers discuss leaching of antimicrobials from food and faeces into the sediments or their subsequent movement to **"*distant sites*"**.

Guardabassi et al. (2000) do not present any analysis of antimicrobial concentrations and cannot, therefore, have any relevance to the issues discussed in the sentence.

Holten et al. (1999) represent a secondary source, which cites two papers as providing data on the presence of antibacterials in water or sediments in the vicinity of fish farms.

Sørum and L'Abée-Lund (2002**)** do not discuss antimicrobial agent use in aquaculture and this paper has no relevance.

Sørum (2000) presents no data on the fate of antimicrobial agents. This paper does provide two sentences commenting on this issue but these comments are not associated with any citation of a primary source,

Sørum (2006) is a secondary source that provides one sentence that relates to the fate of antimicrobials used in aquaculture. This sentence cites a single paper relevant to sediment deposition.

Boxall et al. (2004) provides a detailed review of the literature on the environmental fate antimicrobials used in aquaculture.

Once in the environment, these antibiotics can be ingested by wild fish and other organisms including shellfish (Hektoen et al., 1995; Kerry et al ., 1996; Coyne et al ., 1997; Holten et al ., 1999; Sørum, 2000; Sørum and L'Abée-Lund, 2002; Boxall et al ., 2004; Sørum, 2006).

Four of the papers cited here (Hektoen et al. (1995), Kerry et al. (1996), Holten et al. (1999) and Sørum and L'Abée-Lund (2002) contain no data or information concerning antimicrobial concentrations in '***wild fish and other organisms including shellfish***'.

Sørum (2000) makes an unreferenced comment about the consumption of antimicrobials by wild fish in one sentence.

Sørum (2006) is a review that cites two papers providing primary data on the antimicrobial concentrations in '***wild fish and other organisms including shellfish***'.

Coyne et al. (1997) do provide data on the concentrations of oxytetracycline in mussels in the vicinity of marine fish cages. They reported detectable concentrations in mussels immediately under the cages (with a half-life of approximately 2 days) but failed to detect any in mussels 20 m from the cages.

Boxall et al. (2004) provides a detailed review of the literature on residues in wild fish and shellfish.

These residual antibiotics will remain in the sediment, exerting selective pressure, thereby altering the composition of the microflora of the sediment and selecting for antibiotic-resistant bacteria (Kruse and Sørum, 1994; Hektoen et al., 1995; Davison, 1999; Miranda and Zemelman, 2002a,b; Burrus and Waldor, 2003; Balaban et al., 2004; Beaber et al., 2004; Hasting et al., 2004; Kim et al., 2004; Sørum, 2006).

A total of eleven papers are cited in support of the statements made in this sentence. None of these papers discuss the persistence, of antimicrobials used in aquaculture, in sediments, none discuss the selective pressure exerted by the concentrations of antimicrobials that have been detected in such sediments and none discuss the impacts of these concentrations on the sediment microflora. Equally none of the 11 papers discuss the selection of antibiotic resistant bacteria in impacted sediments.

Kruse and Sørum (1994) addresses transfer of resistance genes in simulated natural environments and does not discuss issues relevant to this sentence.

Hektoen et al. (1995) presents no microbiological data at all.

The papers by Burrus and Waldor (2003), Balaban et al. (2004), Beaber et al. (2004) and Hasting et al. (2004) do not discuss sediment concentrations associated with aquaculture, selection for resistant variants in sediments or impact of antimicrobial on sediment microflora. Many of these papers do address important and interesting issues that would be relevant to a detailed discussion of resistance gene ecology but their contents are not relevant to the comments made in the sentence to which they are attached.

Davison (1999) makes the marginally relevant but unreferenced, comment, "*The spread of antibiotic resistance genes may be causally related to the overuse of antibiotics in human health care and in animal feeds. A similar situation has arisen with bactericides (copper and streptomycin) used in the management of plant disease and with antibacterial agents in intensive fish farms."* This paper does not discuss sediment concentrations.

Miranda and Zemelman, 2002a studied the frequencies of OTC resistance in farms with "*no history of recent oxytetracycline exposure*". Their observations cannot, therefore, relate to selective pressure. Miranda and Zemelman (2002b) presented a further analysis of strains isolated during the survey reported by Miranda and Zemelman (2002a).

Kim et al. (2004) reports the frequency of different *tet* genes in bacteria isolated from Japanese and Korean aquaculture sources. This paper does not provide data that can be used in support of the claim that selection for strains containing these resistances was mediated through concentrations in sediments

Sørum (2006) is a review that cites one paper that provides primary data on sediment concentrations but does not discuss selection in sediments.

There are a number of important studies that indicate that the bacterial flora in the environment surrounding aquaculture sites contain an increased number of antibiotic-resistant bacteria (Huys et al., 2000; Schmidt et al., 2000; 2001a,b; Sørum, 2000; Miranda and Zemelman, 2002a,b; Furushita et al., 2005; Sørum, 2006),

In considering the relevance and appropriateness of the references cited to support the claims made in this sentence, two issues must be born in mind.

1. A large number of studies have reported significant to high levels of resistance in the microflora of relatively pristine, unpolluted waters or water bodies free of known anthropogenic influence. These would include the studies of Kelch and Lee (1978), Jones et al. (1986), Alvero (1987), Bello et al. (1987), Amundsen et al. (1988), EI-Zanfaly et al. (1988)*,* Magee and Quin, (1991), Boon (1992), McKeon, et al. (1995), Boon and Cattanach (1999).
In considering the relevance of the papers cited here, the key issue is, therefore, whether they demonstrate not the presence of bacteria resistant to antimicrobials but to an **increase** in the number of these bacteria.

2. The context of the sentence under consideration indicates that the increases in the number of antibiotic-resistant bacteria being referred to are those that have resulted from the presence of antimicrobials in the environment. There are data demonstrating that increased nutrient loading will increase the frequency with which bacteria resistant to various agents can be isolated from any aquatic environment (Kapetanaki et al. (1995) and Vaughan et al. (1996).
Thus it is essential that the cited references demonstrate that the increases are related to antimicrobial use or that adequate controls are included in the studies reported that such nutrient effects could be accounted for.

Huys et al. (2000) did not study sites with any history of antimicrobial use, did not provide data on the numbers or concentrations of resistant bacteria isolated, did not report data from any control sites and, therefore, could not have reported ***"an increased number of antibiotic-resistant bacteria".***

Schmidt et al. (2000) did study the frequencies of resistant bacteria at four farms and reported on the antimicrobial agent use at these farms. Their data demonstrated, in some cases, that there were higher frequencies of resistance in the ponds and their effluents than in their influents. They failed, however, to demonstrate a correlation between resistance frequencies and use of antimicrobial agents either on a sample-by-sample basis or on a farm-by-farm basis.

Schmidt et al (2001a) only presents a re analysis of isolates collected in Schmidt et al. (2000) and therefore adds no new information relevant to any increase in numbers of resistant bacteria.

Schmidt et al. (2001b) reports studies of resistance genes in isolates of *Aeromonas salmonicida* made from infected fish and the paper has, therefore, only a very marginal relevance to the issues discussed here.

Sørum (2000) is a secondary source that cites the work of Sandaa et al (1992) as a source of data on resistance in bacteria in under-cage sediments. Sandaa et al. (1992) however demonstrated only the presence of resistant bacteria in these sediments after a period of therapy. As they made no observations of the sediment microflora prior to the therapy, their data cannot be taken as demonstrating an increase in the number of antibiotic-resistant bacteria.

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Miranda and Zemelman (2002a) recorded higher numbers of oxytetracycline resistant bacteria in the effluent of four fish farms but these results were obtained from farms that had no history of recent use of this agent. "*Four freshwater Chilean Atlantic salmon (Salmo salar L.) farms with no history of recent oxytetracycline exposure, located at the South of the country were considered in this study. Sampled farms were not exposed to antibacterial therapy for more than 6 months before the sampling period*."

Miranda and Zemelman (2002b) only presents a re analysis of isolates collected in Miranda and Zemelman (2002a) and therefore adds no new information.

Furushita et al., 2005; This citation is to an abstract of a conference paper that I have not been able to access. However, the title "*Analysis of plasmids that can transfer antibiotic resistance genes from fish farm bacteria to clinical bacteria* "does not suggest a study of selection in the environment.

Sørum, (2006) A review of resistance genes and primarily in fish pathogens. The only mention I can find in this paper relevant to selection in sediments is one reporting the work of Schmidt et al. (2000). Thus, this citation represents a secondary source and its citation here is redundant.

and that these bacteria harbour new and previously uncharacterized resistance determinants (Miranda et al ., 2003; Furushita et al ., 2005; Poirel et al ., 2005a,b; Roberts, 2005; Saga et al ., 2005).

Miranda et al. (2003) reported that the PCR primers they used did not amplify material from 10 of the 25 strains of the oxytetracycline resistant bacteria that they studied. It is reasonable to assume that some of these strains may have possessed previously uncharacterised resistance determinants.

Furushita et al. (2005) is an abstract of a conference paper that I have not been able to access.

Poirel et al. (2005a) used a PCR strategy to detect quinolone resistance genes in *Vibrionaceae*. This approach can identify only variants (*qnr*-like genes) of previously characterised resistance determinants. They did not provide any evidence relating the strains they studied to aquaculture.

Poirel et al. (2005b) identified a previously characterised gene (*qnr*A) encoding quinolone resistance in *Shewanella alga* strains isolated from human patients. Although *Shewanella alg*a infections have been associated with seawater contact, Poirel et al. (2005b) do not present data relating the strains they studied to aquaculture.

Roberts (2005) is a secondary source that cites a paper identifying the previously uncharacterised *tet35* in *Vibrio harveyi.*

Saga et al. (2005) identified a variant of known *qnr* genes in a *Vibrio parahaemolyticus* isolated at Tohoku University Hospital, Sendai, Japan. They did not present any data relating this strain to the aquaculture environment.

Aquaculture as a source of antibiotic resistance in human pathogens

Molecular and epidemiological evidence has demonstrated that antibiotic resistance determinants of resistant Salmonella enterica serotype Typhimurium DT104, an

emergent pathogen and the cause of several outbreaks of salmonellosis in humans and animals in Europe and the USA, probably originated in aquaculture settings of the Far East (Angulo, 2000; Angulo and Griffin, 2000; Angulo et al., 2004)

Angulo et al. (2004) is completely irrelevant, as it does not address fish or aquaculture.

Both Angulo (200) and Angulo and Griffin (2001) are secondary sources. Essentially the evidence discussed in them is the same and one or other must, therefore, be considered redundant.

The use of the word ‘probably’ in the above sentence suggests much stronger evidence than is suggested by the use of the word ‘may’ in the cited papers.

Angulo (2000) claimed that the evidence indicated that **some** of the resistance **might** have originated in aquaculture.

Angulo and Griffin (2000) stated, “*Use of antimicrobial agents in aquaculture in Asia **may** have contributed to the emergence of DT104.”*

It is also worth noting that Angulo and Griffin (2000) was a letter to the editor that was written to challenge an alternative model of the emergence of multidrug-resistant *Salmonella* serotype *Typhimurium* DT104 that had been suggested by Davies et al. (1999).

The antibiotic resistance determinants of S. Typhimurium DT104 are encoded by a transmissible genetic element in the chromosome that contains a resistance gene for florfenicol, an antibiotic extensively used in aquaculture in the Far East (Briggs and Fratamico, 1999; Angulo, 2000).

Neither cited paper comments on or provides data on the use of florfenicol in Far Eastern aquaculture. Although it is not cited here, it should also be noted that Angulo and Griffin (2001) make the unreferenced comment that florfenicol was used in Japanese aquaculture from the early 1980s. Information from Schering Plough suggests the first date that they supplied florfenicol to this industry was 1990.

With respect to the genetic structures encoding resistance in DT104, Angulo (2000) is a secondary source that derives its information on the resistance determinants from Briggs and Fratamico (1999) and Bolton et al. (1999).

Although Briggs and Fratamico, (1999) provide evidence for a probable chromosomal location for some of the determinants they do not provide evidence for transmissibility.

This florfenicol determinant, floR, was detected for the first time in the fish pathogen Vibrio damsela (Bolton et al., 1999).

Bolton et al (1999) represent a secondary source and the information it presented with respect to the first isolation of a *flo*R gene has subsequently been shown to be incorrect (Cloeckaert et al., 2001).

A simple search of the literature would have found evidence for the presence of this gene in a bacterium first isolated from a human in 1969 (Chabbert et al., 1972). Meunier et al (2003) also reported the gene as present in a *Salmonella enterica* strain isolated from a turkey in 1990.

These data suggest that *flo* gene variants were circulating in human pathogens well before 1990 when, according to Schering Plough, florfenicol from their company was first used in Japanese aquaculture.

The tetracycline resistance determinant carried by this Salmonella genetic element belongs to the class G that was also, for the first time, detected in the fish pathogen Vibrio anguillarum (Briggs and Fratamico, 1999; Angulo, 2000; Angulo and Griffin, 2000).

Briggs and Fratamico (1999) reported the sequence similarity of part of the tet genes in Salmonella with sequences of the tet(G) in *Vibrio anguillarum* determined by Zhao and Aoki (1992).

Angulo (2000) and Angulo and Griffin (2000 are secondary sources and their use here is redundant as they contain no information not presented in Briggs and Fratamico (1999)

Moreover, the DNA sequence of the transmissible element harbouring these antibiotic resistance determinants has an important DNA sequence similarity to a plasmid of Pasteurella piscicida, which is also a fish pathogen (Kim and Aoki, 1993; Angulo, 2000; Angulo and Griffin, 2000)

Angulo (2000) Angulo and Griffin (2000) are secondary sources and the citation here of one or both is redundant. Both cite Kim and Aoki (1993) and Briggs and Fratamico (1999) as the source of the primary data. The reference provided by GenBank for the *Pasteurella piscicida* sequences to which Briggs and Fratamico (1999) found similarity was Kim and Aoki (1996) and not Kim and Aoki (1993).

This molecular evidence strongly suggests that there was horizontal transmission of antibiotic resistance determinants from bacteria in the aquaculture environment to a human and terrestrial veterinary pathogen (Angulo, 2000; Angulo and Griffin, 2000).

Angulo (Angulo, 2000; Angulo and Griffin, 2000) has argued that the evidence indicates a direction of flow of resistance genes from aquatic bacteria to terrestrial bacteria. This argument is repeated here.

Even if the observation that *flo* and *tet(*G) genes were first identified in aquatic bacteria were correct it would represent only weak support for this position. Such data can be biased by any differential research effort in the study of resistance genes in different bacterial groups. However, as argued elsewhere, a search of the literature, rather than a reliance on secondary sources, would have revealed that *flo* genes had been identified in terrestrial bacteria isolated 23 years before their first detection in aquatic bacteria (see above).

The epidemiology of the dissemination of S. Typhimurium DT104 also suggests this pathogen could have been spread by fishmeal as has happened with the Salmonella Agona that originated in Peru several years ago (Clark et al., 1973; Angulo, 2000; Boyd et al., 2001).

Clark et al. (1973) provides evidence of a role for fishmeal in the epidemiology of salmonellosis. It should be noted that the detection of Salmonella in fishmeal does not represent evidence that those bacteria were derived from the aquatic environment.
In a more rigorous review one might have expected a mention of the work of Nesse et al (2003), which demonstrated the role of contamination during processing of fishmeal, and would, therefore, have been relevant to any serious discussion of this issue.

Again Angulo (200) is an unnecessary, redundant and secondary source.

It is difficult to find anything in Boyd et al. (2001) that suggest any relevance to the spread of *Salmonella* by fishmeal.

For example, V. cholerae of the Latin American epidemic of cholera that started in 1992 appeared to have acquired antibiotic resistance as a result of coming into contact with antibiotic-resistant bacteria selected through the heavy use of antibiotics in the Ecuadorian shrimp industry (Weber et al., 1994; Angulo, 2000).

As the references cited are Angulo (2000) and Weber et al (1994), both of which refer to an epidemic in Ecuador that started in 1991, it must be assumed that the date 1992 is an error.

The Angulo (2000) paper is a secondary source and is redundant in that it cites, with questionable legitimacy, Weber et al (1994) as its sole source of information about this epidemic.

In discussing the possible pressure that might have led to the emergence of multi-drug resistance in Ecuador, Weber et al (1994) discuss three issues.

i) Prior to the main investigation the health authorities had mounted a campaign of prophylactic administration of tetracycline, erythromycin and/or trimethoprim-sulphamethoxazole to those considered at risk.

ii) Antimicrobial agents are available without prescription in Ecuador. Thus, they considered that "*many people may have taken self-prescribed antimicrobial agents as prophylaxis for cholera*."

iii) The third issue that they considered was aquacultural use. They postulate that antimicrobial agents, used in shrimp hatcheries to control non-cholera *Vibrio* infections may have exerted an additional environmental pressure. They do not, however, report the isolation of multi-resistant non-cholera *Vibrio* from these hatcheries. The one strain of *V cholera* isolated from an aquatic animal in this study was, interestingly sensitive to these drugs.

Interestingly, one of these quinolone-resistant determinants has been recently detected in Japan and in Chile in the emergent human pathogen Vibrio parahaemolyticus (Gonzalez-Escalona et al ., 2005; Poirel et al ., 2005a; Saga et al ., 2005; F.C. Cabello and L. Dubytska, unpublished), a marine bacterium transmitted to humans by the ingestion of raw shellfish and that is most likely able to exchange genetic information with other bacteria of the marine environment (Sørum, 2006).

Gonzalez-Escalona et al. (2005) present an epidemiological study o*f V. parahaemolyticus* in Chile but do not mention quinolone resistance.

Both Poirel et al. (2005a) and Saga et al. (2005) reported *qnr*-like genes or *qnr* homologues in *V. parahaemolyticus***.**

A scan of Sørum, 2006 failed to detect any mention of *V. parahaemolyticus*

Additional effects of the excessive use of antibiotics in aquaculture

Another problem created by the excessive use of antibiotics in industrial aquaculture is the presence of residual antibiotics in commercialized fish and shellfish products (Grave et al., 1996; 1999; Goldburg et al., 2001; Cabello, 2003; 2004; Angulo et al., 2004; Sørum, 2006).

Neither Grave et al. (1996, 1999) or Sørum (2006) present any data on residues in commercial fish or shellfish products.

Goldburg et al. (2001) makes an unreferenced mention of residues as a potential risk in a Table. They also mention the World Trade Organisations position on trade limitation based on product safety such as antibiotic residues.

The Angulo et al. (2004) paper not only does not address residues. It does not even address aquaculture.

I have not consulted the two Cabello papers. It should be noted, however, that Cabello (2003) is a review and cannot be a source of primary data.

This problem has led to undetected consumption of antibiotics by consumers of fish with the added potential alteration of their normal flora that increases their susceptibility to bacterial infections and also selects for antibiotic-resistant bacteria (Grave et al., 1996; 1999; Alderman and Hastings, 1998; McDermot et al., 2002; Greenlees, 2003; Cabello, 2004; Salyers et al., 2004)

Grave et al. (1996, 1999) and Alderman and Hastings (1998) do not mention and do not provide data relevant to this issue.

McDermott et al. (2002) is a secondary source for the claim that antimicrobial residues can select for antimicrobial resistance in consumers.

Greenlees (2003) is a review of the implications of antimicrobial drug resistance for the evaluation of human food safety but contains no relevant data, primary or otherwise.

Salyer et al. (2004) discusses the transfer of resistance gene in the human intestine but does not address the impact of any antimicrobial residues in food on these processes.

I have not yet accessed a translation Cabello (2004).

Moreover, undetected consumption of antibiotics in food can generate problems of allergy and toxicity, which are difficult to diagnose because of a lack of previous information on antibiotic ingestion (Alderman and Hastings, 1998; Cabello, 2004).

Alderman and Hastings, 1998 do not discuss the consequences of consumption of antibiotics that might be present in farmed aquaculture products. The only comment they make about residues concerns changes in EU requirements for monitoring such products. Alderman (pers com.) has stated that this comment and the one in the previous quotation represent "*a travesty of what the review actually says*".

I have not yet accessed a translation Cabello (2004).

In aquaculture, the passage into and permanent existence of large amounts of antibiotics in the environment of water and sediments also have the potential to affect the presence of the normal flora and plankton in those niches, resulting in shifts in the diversity of the microbiota (Davies et al., 1999; Miranda and Zemelman, 2001; Cabello, 2003; Hunter-Cevera et al., 2005; Sørum, 2006)

None of the cited papers provide evidence for the *"permanent existence of large amounts of antibiotics in the environment of water and sediment…"*

Davies et al. (1999) is a report of a colloquium. The only (unreferenced) comment it makes that appears to have any slight relevance to the issue being discussed in this sentence is;
"*Antimicrobial agents can be found in sewage effluents, especially in places where they are used extensively, such as hospitals, pharmaceutical production plants, and near farms where animal feed containing antimicrobial agents is used. The contaminated sewage can make its way to streams, lakes and ultimately, the ocean. In a variety of interconnected ecosystems, antimicrobial agents can lead to the emergence of resistance, the reduction of microorganisms susceptible to the agents, and drastic alterations in the biodiversity of affected ecosystems.*"

Miranda, C.D., and Zemelman, R. (2001) is a report of the microbiology of commercially caught fish and has nothing to do with the environmental impacts of antimicrobial agents used in aquaculture.

I can find no reference to the impacts of antimicrobial or antibiotics on diversity in Hunter-Cevera et al. (2005).

Sørum (2006) is a review and the only comment I can find of any possible relevance is to the work of Schmidt et al. (2000).

Cabello (2003) has not been read but is a review and cannot provide primary data.

These shifts can be amplified by the eutrophication produced in the aquaculture environment by the increased input of N, C and P generated by the non-ingested food and fish faeces (Goldburg et al., 2001; Cabello, 2003; Hunter-Cevera et al., 2005)

Goldburg et al. (2001) and Hunter-Cevera et al. (2005) do discuss, in general terms, the relationship of aquaculture and eutrophication. They are, however, colloquium reports and do not, in this regard, provide specific citations to primary data.

Cabello (2003) has not been read but is a review and cannot provide primary data.

Policies of antibiotic use in aquaculture

In analysing the next five quotations from Cabello (2006) it has been assumed that there is a difference in meaning between 'reduction' and 'restriction'.
In these notes 'reduction' in antimicrobial use is taken to mean, as it is in the title of Markestad and Grave (1997), the use of smaller amounts of these drugs over time.
In contrast, 'restriction' is taken to indicate the limitation of use by regulation.

Evidence indicating that antibiotic-resistant bacteria and antibiotic resistance determinants pass from the aquatic to the terrestrial environment has resulted in a drastic restriction of the use of antibiotics in aquaculture in many countries (Markestad and Grave, 1997; Lillehaug et al., 2003; Angulo et al., 2004; Cabello, 2004; Goldburg and Naylor, 2005; Sørum, 2006).

There has not been "***drastic restriction of the use of antibiotics in aquaculture in many countries"***. This statement is incorrect.

Angulo et al. (2004) does not discuss aquaculture or fish farming.

I can find no mention of 'antimicrobials' or 'antibiotics' in Goldburg and Naylor (2005).

I have not accessed a translation of Cabello (2004).

The remaining three citations are to papers that discuss antimicrobial agent use in Norway. In this country there was a dramatic, and well documented, reduction in antimicrobial agent use during the 1990s.

Markestad and Grave (1997) state "*Introduction of oil-adjuvanted vaccines has been the single most important cause for the substantial reduction in use of antibacterial drugs in Norwegian fish farming industry*." This paper does make a general and unreferenced comment that the use of antibacterial drugs is *"directly linked to the emergence of drug-resistant bacteria"* but does not discuss transfer of these bacteria or their genes to the terrestrial environment.

Lillehaug et al. (2003) report the reduction in antimicrobial use in Norway between 1991 – 2000 but, it is clear from this paper, that the reduction was a consequence of the reduction of disease incidence and not as a result of restrictions introduced to address concerns about resistance transfer to the terrestrial environment.

Sørum (2006) only makes a passing reference to the reasons underlying the *reduction* of antimicrobial use in Norwegian Atlantic salmon farming. He implies that effective vaccines were the most significant factor. He further suggests that the continued high levels of use of these agents in Chile is a consequence of the absence, so far, of an effective vaccine against *Piscirickettsia salmonis* an important pathogen in this country.

Restrictions have included, increased control of the prescription of therapeutic antibiotics (Grave et al., 1996; 1999; Markestad and Grave, 1997; Lillehaug et al., 2003; Sørum, 2006), …..

All five papers cited here relate to Norwegian experience.

Grave et al. (1996) and Lillehaug et al. (2003) mention changes in the law concerning prescribing of antimicrobial agents for aquaculture in Norway but these changes did not include any restrictions regarding use of specific antimicrobials.

Grave et al. (1996) reported restrictions aimed at reducing on-farm-mixing of medicated feeds.

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According to Lillehaug et al. (2003) changes, which largely involved administrative reporting procedure and not patterns of prescribing, were introduced to facilitate residue testing.

Markestad and Grave (1997) do not discuss prescription or mention regulations governing prescription

The claim that there has been an increased control on prescription of therapeutic antibiotics is not supported by any comments made by Grave et al. (1999) or Sørum (2006).

Grave et al. (1999) mentions a relaxation in Norwegian drug regulations that were introduced as a consequence of the European Economic Area agreement in 1994. They report that these changes led to an increase in the number of new substances and preparations approved for veterinary use in Norway.

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…..almost total elimination of the use of antibiotic prophylaxis in this setting (Grave et al., 1996, 1999; Sørum, 2006)

As discussed above, neither Grave et al. (1996, 1999) nor Sørum, 2006) provide support for the claim that (in Norway) the use of prophylactic use of antimicrobials was ever practiced.

Rather than identifying "restrictions" as the cause of the reduction of antimicrobial use in Norwegian aquaculture, Grave et al. (1999) report that the reduction in the use of quinolones and tetracyclines has "*almost solely been due to the use of oil-adjuvanted vaccines in furunculosis."*

and proscription of the use of antibiotics in therapeutics that are still very useful in the therapy of human infections (Grave et al., 1996; 1999; Markestad and Grave, 1997; Goldburg et al., 2001; Lillehaug et al., 2003)

Four of these five citations are to papers that discuss antimicrobial agent use in Norway. There has been no ***proscription*** of the use of these agents by any Norwegian regulatory agency, for any reason.

Grave et al. (1996, 1999) Markestad and Grave (1997) and Lillehaug et al (2003) do not mention any proscription.

Goldburg et al. (2001) mentions the two antibiotics approved by the FDA for use in US aquaculture. It provides no comment, however, relating to the proscription of any agents.

In this way, the use of quinolones has been totally restricted in aquaculture in industrialized countries, not only because they are a highly effective group of antibiotics for human infections but also because of their ability to generate cross-resistance among all the members of this group (Grave et al., 1996; 1999; Gorbach, 2001; Cabello, 2004; Moellering, 2005; Sørum, 2006).

The statement " ***the use of quinolones has been totally restricted in aquaculture in industrialized countries"*** is factually incorrect. Oxolinic acid is currently registered for use in aquaculture in six European countries and flumequine in five.

Neither Grave et al. (1996, 1999) or Sørum (2006) discuss the existence of any regulatory restrictions on the use of quinolones in aquaculture let alone any reasons for such restrictions.
Grave et al. (1996) reported that during the period 1984-1994 quinolones were the most frequently prescribed drug in Norwegian aquaculture.
Grave et al. (1999) reported the use of quinolones in Norway in 1996, the last year for which the present any data.

Gorbach (2001) is an editorial that argues that because of their value in humans the use of fluoroquinolones **should** be prohibited in animals. This paper mentions neither aquaculture nor cross-resistance.

Moellering (2005) is not a research paper or a review. It is simply an introduction to a set of reviews by other authors and contains no data.

I have not yet accessed full text translation of Cabello (2004)

…indicating that it is economically feasible to develop a productive aquaculture industry without excessive prophylactic use of antibiotics (Grave et al., 1996; 1999; Markestad and Grave, 1997; Lillehaug et al., 2003; Sørum, 2006)

The Norwegian experience clearly demonstrates that large scale salmon production can be achieved without excessive use of antimicrobials. However, as discussed above, neither Grave et al. (1996, 1999), Markestad and Grave (1997) nor Sørum, 2006) provide support for the claim that (in Norway) the use of prophylactic use of antimicrobials was ever practiced.
This absence of prophylactic use in Norway is underlined by the data of Lillehaug et al. (2003) who examined 5439 administrations of antimicrobials in Norway between 1991 and 2000 and classified one prescription as for prophylactic use.

Similarly in China, quinolone resistance has emerged as an important public health problem as result of the unrestricted use of this group of antibiotics in aquaculture and in industrial animal husbandry (Wang et al., 2001; Jacoby, 2005).

The existence, in China, of a causal link between aquacultural use and the emergence of quinolone resistance in that country is not supported by either cited paper.

Wang et al. (2001) do not mention the role of **"***unrestricted use of this group of antibiotics in aquaculture"* in the emergence of quinolone resistance problems in China. The words 'fish" or 'aquaculture" do not occur in this paper.

Jacoby (2005) makes no reference to aquaculture or to its role, in China, in the emergence of quinolone resistance.

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Appendix 1

In preparing these notes it has been assumed that papers cited in a review should normally represent primary sources of data but that, on occasions, the use of secondary sources may be legitimate.

Primary sources, normally research papers, should provide the data, and details of the methods used to obtain that data, presented in the review or that has been used to arrive at the conclusions presented in the review. Primary sources may also be used to support an argument or conclusion presented in a review. Primary sources may also provide conclusions or attributions of meaning but only if the primary data is also presented in that paper.

Review articles may have value as secondary sources particularly when they are centred on the specific topic under discussion in the review that cites them. In addition research papers or reviews may have value as secondary sources when the relevant comments observations, or arguments they contain are themselves based on specified and cited, primary sources.

Secondary source that provide general comments or observations that are themselves not supported by references to primary sources can only provide very weak support for comments in a review (see marginal below).

Relevant

Citations to primary source research papers were classified as relevant if the cited paper contained;

i) the data referred to in the text (and the methods used to obtain that data).
or
ii) data relevant to or that provides support for, arguments or statements being made in the portion of review with which they were associated
or
ii) conclusions drawn from primary data themselves presented in the cited paper that were then reproduced in the review.

Citations to secondary sources, such as reviews, were considered relevant if they presented data, conclusions or arguments relevant to those presented in the review if and only if, they provided a reference to the primary source of the data they used.

Marginally relevant

Citations were classified as marginally relevant when they were to data, conclusions or arguments presented in a secondary source that did not provide a citation to the ultimate primary source.

Partially relevant

In writing this review the author frequently makes two or more related observations or statements in one sentence and then cites a number of publications

at the end of that sentence. In these situations it is often not clear to which observation any of the cited papers were supposed to relate.
Citations were classified as partially relevant when they were relevant (see above) to only part of the text to which they were linked by the syntax of the text.
In these notes citations were also classified as partially relevant when the wording in the review represented a simplification or partial treatment of the data presented in the cited paper.

Redundant

As stated above, a secondary source was considered as 'relevant' only if the information it contained was associated with an appropriate reference to the primary source. However, in situations where a primary source had already been cited, the citation of a secondary source that itself had cited the same primary source, was considered redundant.

Not relevant

Citations were classified as not relevant if they failed to meet any of the above criteria.