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Fish feed as a source of oxytetracycline-resistant bacteria in the sediments under fish farms

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Abstract

Concentrations of oxytetracycline and the frequency of oxytetracycline resistance in the environmental microflora were monitored following the therapeutic use of this agent at a marine fish farm. 529 kg of oxytetracycline were administered over a 24 day period at an average of 1.4 kg per cage per day. Three days after the end of the therapy $4.6 \pm 3.7 \mu\text{g/g}$ oxytetracycline were detected in the sediments and the frequency of resistance in the sediment microflora was $9.0 \pm 5.3\%$. A rise in the frequency of resistance in this flora to $26 \pm 8.7\%$ occurred 24 days after the therapy. This rise was not associated with any increase in the concentrations of oxytetracycline in the sediment. At this time the frequency of resistance in the flora isolated from mussels suspended above the sediments ($36 \pm 8.5\%$) was significantly ($P=0.005$) higher than that present in the sediment flora. The feed used on the farm 24 days after the end of therapy was shown to contain 4.6×10^4 oxytetracycline-resistant cfu/g. The distribution of phenotypic groups in the oxytetracycline-resistant flora in this feed and in the sediments during the peak in resistance were compared with those from other marine environments. These data demonstrated that resistant flora in feed can, under certain circumstances, significantly contribute to the resistant flora detected in sediments under fish cages.

Keywords: Oxytetracycline; Feeding and nutrition—fish; Sediments; *Salmo salar*; Environmental impact.

1. Introduction

Nygaard et al. (1992) demonstrated that an increase in the frequency of oxytetracycline-resistant microflora can be selected in the presence of oxytetracycline in marine sediments.

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Following the addition of 50 ppm oxytetracycline to sediment in trays placed on the sea bottom, they demonstrated an increase in resistance, from $5 \pm 1\%$ to $16 \pm 1.5\%$, over a 12-month period. Using aquaria, Hansen et al. (1993) also demonstrated selection in marine sediments. They found that 400 ppm oxytetracycline selected a resistance frequency, to 25 $\mu\text{g}/\text{ml}$ oxytetracycline, of 13.5% after 7 days. After 185 days this frequency had risen to 35.1%.

In field studies the use of oxytetracycline in marine fish farms has been shown to coincide with the presence of elevated frequencies of resistant bacteria in the sediments under the fish cages (Torsvik et al., 1988; Samuelsen, 1989; Lunestad, 1992; Kerry et al., 1994). Samuelsen et al. (1992) reported frequencies of resistance, to 25 $\mu\text{g}/\text{ml}$ oxytetracycline, of over 100% in the sediments under 3 fish cages 12 days after therapy with this agent. These sediments contained 26, 189 and 285 ppm oxytetracycline. No simple correlation between sediment oxytetracycline concentrations and the frequency of resistance was, however, observed during this work. Kerry et al. (1994) also reported a lack of correlation between resistance frequencies and oxytetracycline concentrations. They noted that, following oxytetracycline therapy, elevated frequencies of resistance were found in sediments where no oxytetracycline could be detected. These results raise questions as to the causal relationship between the presence of the oxytetracycline and the elevated frequencies of resistance found in sediments under fish cages.

This work was prompted by an unexpected elevation in the frequency of resistant microflora in the sediment beneath a marine fish farm, 24 days after the end of therapy, in the absence of any rise in detectable oxytetracycline. No attempt was made to classify to a generic level the individual strains comprising this oxytetracycline-resistant microflora. Rather the resistant flora of a variety of environments were characterised by the determination of the frequency within them of broad phenotypic classes of bacteria. This analysis was used to investigate the possibility that the microflora of the non-medicated fish food being fed at that time was the origin of the elevated frequency of resistance detected in the sediments.

2. Materials and methods

Sampling sites

Farm A is located in Galway Bay at $53^{\circ}15.95'\text{N}$, $09^{\circ}44.3'\text{W}$. Both the fate and impact of oxytetracycline following its use at this farm on previous occasions have been reported (Coyne et al., 1994; Kerry et al., 1994). The last use of oxytetracycline at the farm was completed 156 days prior to the start of the experiments reported here and the sediment conditions at this time were similar to those reported by Coyne et al. (1994). During the experimental period the farm consisted of a 16-cage (15×15 m) TurmicTM block stocked with 179 tonnes of Atlantic salmon (*Salmo salar* L.) of average weight 1.4 kg. The average stocking density was 11 ± 2.5 tonnes per cage ($7.3 \text{ kg}/\text{m}^3$). A treatment, with orally administered oxytetracycline, was initiated in response to an outbreak of furunculosis. The medicated feed contained 3.2% w/w oxytetracycline that had been surface-coated on to the pellets by the feed manufacturer. Immediately after the start of treatment, the feeding response of the fish fell rapidly to approximately 50% of normal values and only recovered

slowly during treatment. As a result, treatment was continued for 24 days. The prescribed dose was exceeded and, in total, 529 kg of oxytetracycline were used during the treatment at an average of 1.4 kg/cage per day. Cage 64 was in the centre of the cage block and was stocked with 7.7 tonnes and received 28.9 kg oxytetracycline. The therapeutic treatment was initiated in July when the water temperature was 13–15°C.

Fish farm B was in the same bay as farm A but under different management. At the time of the investigation it was stocked with 10.9 tonnes of Atlantic salmon in 3 Polar Circle™ cages at a stocking density of 1.65 kg/m³. The oxytetracycline treatment at this farm was carried out over 16 days in June when the water temperatures were 15°C. The treatment involved the use of 70 kg of oxytetracycline at an average of 1.5 kg/cage per day.

Non-fish-farm sites C and D were selected as sites at least 5 km from any fin fish farm and free from any known anthropogenic influence.

Sampling

Samples of marine sediments from farms A and B and non-farm sites C and D were collected as described by Coyne et al. (1994). A 2-cm slice from the surface of each core was divided and one half was analysed for oxytetracycline (Coyne et al., 1994) while the other half was analysed for the frequency of resistant bacteria (Kerry et al., 1994).

At fish farm B 10 sediment samples from directly under the cages were collected on the day following the end of therapy. At non-fish-farm sites C and D 9 sediment samples were collected during June when the water temperature was 13–15°C. The sediments under fish farm A were sampled at 3, 6, 10, 17, 24, 30, 38 and 45 days after the end of therapy. On each day, sediment cores were collected from 6 sample sites distributed under the whole cage block and from a further 5 sites under cage 64.

The mussels collected from fish farm A were taken from under cage 64. They were growing on mooring ropes approximately 1 m above the sediment. These mussels were sampled each time sediment samples were collected with the exception of day 45 when insufficient material was collected. The 6 samples of mussels analysed to establish the background frequency of resistance to oxytetracycline were collected from mussel farms in the Galway Bay area. These mussel farms were also at least 5 km distant from any fin fish farm and were free of any detectable mammalian faecal contamination (Neptune Laboratories, unpublished results).

Samples of fish feed were collected from farm A 24 days after the end of therapy. These samples were collected from the feeding bins on the farm. Samples of fish feed were also collected from 16 randomly selected, unopened bags of feed present on fish farms in the Galway area. All the feeds sampled, which had been produced by 3 separate manufacturers, were supplied as non-medicated feed.

Oxytetracycline analysis

Sample preparation and HPLC analysis for oxytetracycline in sediments were performed by the method of Coyne et al. (1994). Samples of mussels were extracted by the method of Samuelsen (1989) and analysed by the HPLC method of Coyne et al. (1994). Oxytetracycline standard curves had a high degree of linearity ($r^2 > 0.99$). The mean recovery of oxytetracycline from sediment samples was $89.0 \pm 4.5\%$ and from mussels was 65%. The limit of detection of oxytetracycline was 0.1 $\mu\text{g/g}$.

Determination of resistance frequencies

Mussel samples were processed by the method of West (1988). Samples of fish feed, sediment and processed mussels were analysed for the frequency of oxytetracycline resistance by plating on 2216 V medium (Zobell, 1941) (ZV) with and without the addition of 25 µg/ml oxytetracycline (Kerry et al., 1994). Analysis of fish feed showed a high degree of variability with respect to both total culturable organisms and the frequency of resistance. For this reason, a minimum of 6 and a maximum of 12 independent samples were analysed for each batch of feed.

Isolation and characterisation of oxytetracycline-resistant bacteria

Colonies were picked at random from plates of ZV agar containing 25 µg/ml oxytetracycline and were restreaked on the same medium until pure cultures were obtained. Culture purity was checked by Gram stain. Pure cultures were stored at 4°C on slopes of Zobells 2216 V agar and 6% aged seawater nutrient agar with oxytetracycline at 25 µg/ml. The pure strains grown on aged seawater nutrient agar were used for the oxidase test as this medium contained no glucose and low nitrate levels, both of which interfere with the oxidase test if present (Cowan, 1974; Eklund and Lankford, 1967). Yeasts were identified by cell morphology, the presence of budding and the Gram reaction. The Gram reaction of bacterial strains was determined using both the Gram stain (Cowan, 1974) and the KOH method (Buck, 1982), oxidase by the method of Cowan (1974) and the oxidative/fermentative (O/F) test was carried out according to Leifson (1963). Motility was determined using phase contrast microscopy and by observing growth on semi-solid agar (Eklund and Lankford, 1967; Cowan, 1974) and strains were considered motile if either of these tests gave positive results. A panel of control bacteria (*Staphylococcus aureus* NCIMB 6571, *S. aureus* NCIMB 9518, *Bacillus subtilis* NCIMB 3610, *Pseudomonas aeruginosa* NCIMB 8295, *Aeromonas salmonicida* NCIMB 1102, *Escherichia coli* K12 NCIMB 10214 W.T. and *Vibrio fischeri* NCIMB 1149) were included each time a set of characterisation tests was performed.

Comparison of the phenotypic distribution of the oxytetracycline-resistant flora from different environments

Colony-forming units were isolated from 7 environments (Table 1). Each isolate was characterised with respect to Gram stain, motility, oxidase and the O/F test. On the basis of these tests, each bacterial isolate could be placed into one of 24 groups. All yeasts were placed in a separate group giving a total of 25 groups (Table 1). Spearman's correlation coefficients were used to calculate the similarity of the distribution of the flora of each environment between these 25 phenotypic groups. Hierarchical clustering of the phenotypic distributions of the resistant flora of these environments was performed using the unweighted pairs group method with arithmetical averages (Priest and Austin, 1993) on these correlation coefficients (Fig. 3).

Table 1

Percentage distribution between 25 phenotypic classes of the oxytetracycline-resistant isolates from 7 environments

Group	Code ^a	Environments ^b						
		1	2	3	4	5	6	7
1	- + +F	4	8	4	15	1	20	12
2	- + +O	11	45	4	66	15	21	30
3	- + +I	5	12	2	7	0	6	9
4	- + -F	3	0	0	0	0	5	1
5	- + -O	2	0	13	0	11	2	2
6	- + -I	2	0	2	0	0	0	0
7	- - +F	2	4	0	0	3	3	5
8	- - +O	6	3	4	0	1	1	1
9	- - +I	6	6	0	0	0	2	8
10	- - -F	1	1	4	0	3	1	1
11	- - -O	1	1	13	0	26	0	0
12	- - -I	1	3	2	0	0	1	1
13	+ + +F	5	0	4	1	0	11	4
14	+ + +O	0	0	2	0	0	1	2
15	+ + +I	1	0	0	1	0	0	2
16	+ + -F	3	1	2	0	7	6	3
17	+ + -O	2	0	11	0	10	3	5
18	+ + -I	0	0	0	0	1	0	0
19	+ - +F	0	1	4	1	4	5	2
20	+ - +O	0	1	4	1	0	0	0
21	+ - +I	0	0	0	0	0	0	1
22	+ - -F	11	5	0	0	0	8	6
23	+ - -O	14	0	23	0	14	1	2
24	+ - -I	1	0	4	1	3	4	4
25	Yeast	23	11	0	9	0	2	2
Number of isolates		127	103	53	116	73	106	113

^aThe results of the Gram reaction, motility, oxidase and the O/F test are given in that order.

^bDescriptions of tested environments: (1) Under-cage sediment from farm A before therapy, 156 days after last use of oxytetracycline in farm ($n = 127$). (2) Under-cage sediment from farm A 3 days after therapy ($n = 103$). (3) Under-cage sediment from farm A 24 days after the end of therapy ($n = 53$). (4) Under-cage sediment from farm B 1 day after the end of therapy ($n = 116$). (5) Fish food being fed at farm A 24 days after the end of therapy ($n = 73$). (6) Sediment from site C over 5 km from any fish farm ($n = 106$). (7) Sediment from site D over 5 km from any fish farm ($n = 113$).

3. Results

Analysis of sediments from sites B, C and D and farmed mussels

The oxytetracycline concentration of these sediments was not analysed. The frequencies of oxytetracycline resistance at the farm site B was $4.9 \pm 0.2\%$ ($n = 10$) and at the two non-farm sites C and D were $1.0 \pm 0.8\%$ ($n = 9$) and $1.2 \pm 1.7\%$ ($n = 9$) respectively. The

frequency of resistance in the 6 samples of mussels taken from mussel farms was $3.0 \pm 1.5\%$.

Analysis of sediment under the cage block at farm A

The mean concentration of oxytetracycline and the mean frequency of oxytetracycline-resistant colony forming units detected in the 6 samples taken from under the whole cage block at farm A are shown in Fig. 1.

Prior to treatment, no oxytetracycline was detected in the sediment. Three days after the end of therapy $4.6 \pm 3.7 \mu\text{g/g}$ ($n=6$) was detected, and 6 days after the end of the therapy the concentration had fallen to $2.4 \pm 1.4 \mu\text{g/g}$ ($n=6$). Concentrations of oxytetracycline did not fall significantly between 6 and 45 days after the end of therapy ($P=0.75$).

Three days after the end of the therapy the frequency of resistance increased to $9.0 \pm 5.3\%$. The frequency then rose significantly ($P=0.004$) to $19.2 \pm 4.2\%$ 6 days after the end of therapy. The frequency declined exponentially ($r^2=0.99$) to $3.0 \pm 1.9\%$ 17 days after the end of therapy. The half-life of this decline was 4 days. Twenty-four days after the end of therapy the frequency again showed a significant rise ($P<0.001$) to $25.7 \pm 8.7\%$ and then fell again exponentially ($r^2=0.99$) to $1.8 \pm 0.91\%$ after 45 days. The half-life of this decline was 6 days.

Analysis of samples from under cage 64

Oxytetracycline concentrations were not determined in the sediment samples taken from under cage 64. In the mussel samples taken from under this cage, the only sample in which oxytetracycline was detected was that taken 3 days after the end of the treatment. At this time the concentration was $1.4 \mu\text{g/g}$. At all other times the concentration was below the limit of detection.

The frequencies of resistance to oxytetracycline in the sediment and mussel samples collected under cage 64 are shown in Fig. 2. On no occasion were the frequencies in the 5 sediment samples from under cage 64 significantly ($P>0.05$) different from those in the 6 samples taken from under the whole cage block. The frequencies of resistance to oxytet-

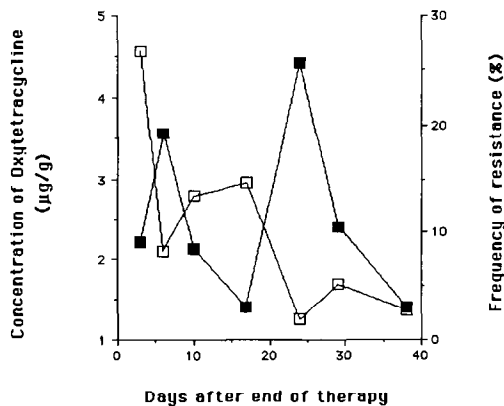


Fig. 1. Concentration of oxytetracycline ($-\square-$), and the percentage of oxytetracycline-resistant microflora ($-\blacksquare-$), in samples taken from under the cage block.

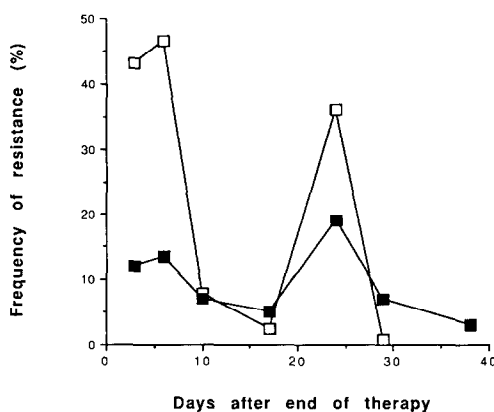


Fig. 2. Percentage of resistant microflora in samples of sediment (-■-), and mussels (-□-), taken from under cage 64.

racycline in flora isolated from mussel samples taken at farm A showed similar fluctuations, with respect to time, to those detected in both sets of sediments taken from farm A. Peak resistance frequencies of $46 \pm 12\%$ were detected 6 days after the end of therapy. The frequencies then fell exponentially ($r^2 = 0.93$) to $2.4 \pm 0.7\%$ 17 days after the end of therapy. The half-life of this decline was 3 days. Twenty-four days after the end of therapy a second peak in the frequency of $36 \pm 8.5\%$ was detected. This fell to $0.7 \pm 0.7\%$ 5 days later. The peak frequencies of resistance in mussels sampled on both the 6th and 24th day after therapy were significantly higher than those detected in the sediments on the same days ($P < 0.001$ and $P = 0.005$, respectively).

Oxytetracycline and resistant bacteria in fish feed

The concentration of oxytetracycline in the non-medicated fish foods was not determined by HPLC analysis. The concentration of resistant colony-forming units (cfu) in samples ($n = 12$) of the feed being fed to the fish in farm A, 24 days after the end of therapy was $4.6 \times 10^4/\text{g}$. This represented 123% of the total colony-forming units detected. These parameters were also determined for 16 other commercial feeds. In 9 of the feed samples the levels of total and resistant colony-forming units were less than the limit of detection ($< 10^3 \text{ cfu/g}$). In the remaining 8 feeds the concentration of resistant colony-forming units ranged from 3.3×10^3 to $2.6 \times 10^4/\text{g}$ (median $7.3 \times 10^3/\text{g}$) and the frequency of resistance ranged from 7 to 65% (median 15%).

Characterisation of oxytetracycline-resistant flora

Oxytetracycline-resistant colonies were isolated from the 7 different environments detailed in Table 1. The flora isolated from the mussels 24 days after therapy were, unfortunately, not included in this analysis. The hierarchical cluster analysis of the floral distribution from each environment (Fig. 3) revealed two groups of environments which had unrelated distributions of oxytetracycline-resistant flora ($P > 0.05$). The first group consisted of the two non-fish-farm environments and the sediments at farm A prior to the use of oxytetracycline. The relationship between the distribution of the phenotypic classes in

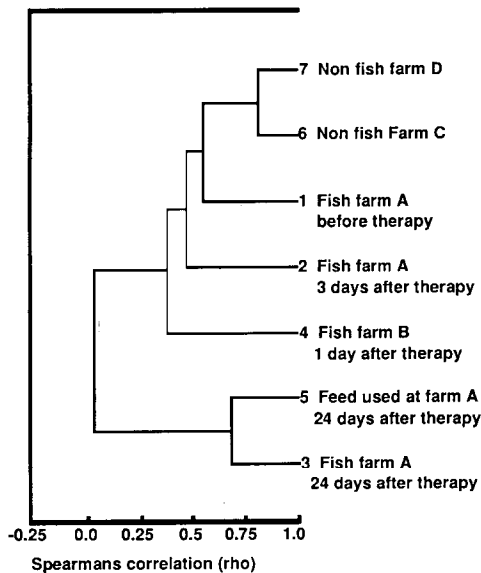


Fig. 3. Dendrogram of the correlations of the distribution between 25 phenotypic classes of the oxytetracycline-resistant flora isolated from 7 environments. The dendrogram was calculated using the unweighted pair group method with arithmetic averages. The environments are as listed in Table 1.

these environments was highly significant ($P < 0.01$). At a slightly lower level of significance ($P < 0.05$) this group also included the sediments at fish farms A and B 3 and 1 days after the end of therapy, respectively. The second group, which showed no relationship with the first, consisted of the fish food and the sediment of farm A 24 days after the end of therapy.

4. Discussion

Considerable numbers of oxytetracycline-resistant micro-organisms were detected in some fish feeds in this work. It should be noted that the feed bags that were examined were those present on the cages on the day of sampling. The data presented are therefore only relevant to the quality of feed as it is presented to fish in commercial fish farms. It does not provide information on the quality of the feed as it left the manufacturers or on the influence of the various storage times and conditions used by farmers. Initial examination of these data suggests that this level of contamination may present serious problems for fish husbandry. Administration of the feed used at farm A 24 days after therapy, for example, would result in daily inputs of 8.0×10^5 resistant organisms to a 1.4-kg fish feeding at 1.5% body weight per day. It should be noted, however, that it is unlikely that the majority of the oxytetracycline-resistant micro-organisms in the feed were pathogenic for fish. On the other hand, to the extent that their oxytetracycline resistances are plasmid-mediated, they may be able to transfer such resistance to pathogens resident in the fish intestinal tract or in under-cage sediments. Such transfer has not been experimentally confirmed in this work. Sandaa

et al. (1993) have shown, however, that plasmid transfer can occur in the environment of fish farms and is favoured by the presence of a selective antimicrobial agent.

The half-lives of the elevated frequency of resistance detected in the sediment 24 days after the end of therapy suggested that these, feed-derived, micro-organisms can not survive for long periods in the marine environment. The data can not provide accurate estimates of these survival rates. The half-life of the peak of elevated resistance detected in this work may well have been influenced by the microflora of the batches of feed used in the days following day 24. These batches were not analysed. It should be noted that the size of the farm studied and the feed management protocols in operation result in a fast turnover of feed at this farm. In practice, a single production unit, 2 tonnes, is rarely fed for a period of greater than 2–3 days. The factors that lead to the detection of significant numbers of oxytetracycline-resistant micro-organisms in the commercial feeds, at the time they are being fed to fish, have not been investigated in this work. Clearly both the origin of these organisms and their significance require urgent study.

The concentration of oxytetracycline ($4.6 \pm 3.7 \mu\text{g/g}$) and the frequency of oxytetracycline resistance ($9.0 \pm 5.3\%$) detected 3 days after the end of therapy at farm A can be compared with the data previously published concerning therapy at this farm (Coyne et al., 1994; Kerry et al., 1994). Although the therapy studied in this work continued for a longer period and a greater amount of oxytetracycline was used, the concentrations of oxytetracycline and oxytetracycline-resistant microflora were of the same order of magnitude as those previously reported at this site. These data, therefore, confirm that the therapeutic use of oxytetracycline at this farm has dramatically different consequences from its use at the farm studied by Samuelsen et al. (1992) who reported oxytetracycline concentrations of 285 and 189 ppm, and resistance frequencies of $> 100\%$ under treated cages. The frequency of resistance of $4.9 \pm 0.2\%$ at fish farm B reported in this work demonstrates that, with respect to this parameter, the results obtained from farm A are not unique to that farm.

Significant rises in the frequency of oxytetracycline resistance, in the under-cage sediment microflora, were detected between 3 and 6 days after the end of the therapy ($P=0.004$) and between 17 and 24 days after the end of therapy ($P<0.001$). The concentrations of oxytetracycline detected in the under-cage sediments provide no obvious explanation for these dramatic rises in frequency (Fig. 1). This raises the possibility that the selection for the observed increase in the frequency of resistant microflora did not occur in the sediment. Samples taken from under cage 64 exhibited similar peaks in resistance frequency in both sediment and mussel samples. The frequencies of resistance were, on both occasions, significantly higher in the mussels growing above the sediments than in the sediments themselves ($P<0.001$ and $P=0.005$, respectively). As oxytetracycline was detected only in those mussels sampled 3 days after therapy these high frequencies of resistance, particularly those detected on 24 days after therapy, cannot be accounted for by selection resulting from oxytetracycline in the mussels themselves (Fig. 2). Rather these data suggest that the resistant microflora originated from above the mussels and therefore the most plausible sources were the faeces or the feed falling from the cages. Increases in the frequency of oxytetracycline resistance in faecal and gastrointestinal microflora of fish following oral oxytetracycline therapy have been reported (Austin and Al-Zahrani, 1988; Sugita et al., 1988; Björklund et al., 1990, 1991). Neither fish faeces nor their intestinal contents were collected during this experiment. The feed used 6 days after the therapy was available, but

had been stored at -20°C . Storage at this temperature significantly reduces the number of bacteria which can be isolated from the feed (unpublished results). For this reason no analysis was made of the resistance frequency of the flora present in the feed used at this time. The feed used on farm A 24 days after the end of therapy, during the second significant rise in the frequency of resistance in the sediment and mussel microflora, was available for analysis. This feed was shown to contain a significant number of micro-organisms of which a high percentage (> 100) were resistant to oxytetracycline. A frequency of resistance of over 100% is, upon superficial examination, absurd and may raise questions as to the suitability of the method used to generate the data. No investigations are reported here into this apparent absurdity, but it is noted that frequencies of over 100% were also reported by Samuelson et al. (1992). The number of resistant micro-organisms cultured from the feed ($4.6 \times 10^4/\text{g}$), however, means that the feed represents a potential source of the resistant micro-organisms detected in the sediment and the mussels. To test this hypothesis, the resistant flora present in the sediment samples taken 24 days after the end of therapy were compared to those present in the feed used at that time and to those isolated from other marine environments.

The results of this analysis (Table 1, Fig. 3) show that the floral distribution present in the sediment 24 days after therapy (environment 3) shows dissimilarities to those found in two farms sampled following oxytetracycline therapy (environments 2 and 4). The floral distribution was also dissimilar to those found in undisturbed marine sediments (environments 6 and 7) and in the fish farm sediment 156 days after the last use of oxytetracycline (environment 1). Thus the population structures of micro-organisms that contribute to the peak frequencies of resistance detected 24 days after the end of therapy are unrelated to those found in all other marine sediments tested.

The small number of characterization tests used in the analysis, however, has a consequence that similarities in the phenotypic distribution of the flora of two environments must be given less significance than dissimilarities. The analysis did, however, detect a very high degree of similarity ($r=0.81$; $P<0.001$) between the flora of the two non-fish-farm sediments (environments 6 and 7). The flora of these two environments would be expected to be similar as neither was subject to any identifiable input that might be expected to cause a modification of their microflora from the distribution typical of the sediments of Galway Bay in general. Thus, the high degree of similarity detected between the flora of the fish feed and that isolated from the sediments at fish farm A, 24 days after the end of therapy ($r=0.68$; $P<0.001$) may be taken as having some significance.

In terms of the specific phenotypic groups isolated, there were significant differences between the resistant flora from fish farms A and B immediately after therapy (environments 2 and 4) and those from farm A 24 days after therapy and the fish food used at that time (environments 3 and 5). The dominant groups in environments 2 and 4 were Gram-negative, motile and oxidase-positive, and were either inert or oxidative with respect to the O/F test. All *Pseudomonas* isolated would have been placed in these two groups (Shewan et al., 1960). In environments 3 and 5 these two groups were significantly ($P<0.001$) less frequent (6 and 15%, respectively). In contrast, oxidase-negative isolates with an oxidative attack on glucose dominated the flora of environments 3 and 5 (60 and 61%) and were almost completely absent from the flora of environments 2 and 4 (1 and 0%, respectively). These changes show similarities to those reported by Austin and Al-Zahrani (1988) in the

gastrointestinal flora of rainbow trout following the oral administration of oxytetracycline. They reported that therapy resulted in a reduction in the frequency of *Pseudomonas* sp. (Gram-negative, oxidase-positive and oxidative or inert) and an increase in *Flavobacterium* sp. (Gram-negative, oxidase-negative, and oxidative) and Coryneforms (Gram-positive, oxidase-negative and oxidative). Austin and Al-Zahrani (1988) did not report any examination of the feed used in their experiments, but the data presented above raise the intriguing possibility that the feed may have been the source of the resistant flora they detected.

In conclusion, this paper presents a variety of evidence relating to the probable source of the flora contributing to the peak in the frequency of oxytetracycline resistance detected in sediments 24 days after the end of therapy. The peak in resistance frequency was not preceded by any increase in oxytetracycline in the sediments. The highest frequencies of resistance occurred in the mussels above the sediments rather than in the sediments. The half-life of the elevated frequency was short, suggesting that the micro-organisms responsible were not capable of colonising the sediment. The phenotypic distribution in the resistant flora during the peak was dissimilar to that of the resistant flora isolated from 5 other marine sediments but similar to that of the flora isolated from the feed used at that time. The dominant phenotypic groups present in the sediments at this time were not dissimilar to those reported to be present in the intestine of rainbow trout following oral therapy (Austin and Al-Zahrani, 1988). Taken together, these data strongly support the hypothesis that the resistant flora introduced in the feed were the most probable source of the elevated frequency of resistance in the sediments 24 days after therapy. The origin of the elevated frequency of resistance detected in the sediment 6 days after therapy was not investigated in as much detail. Some of the above arguments can, however, also be applied to this event and it is possible that the flora present in the feed may also have contributed to this rise.

No data or arguments are presented in this paper suggesting that the presence of oxytetracycline in sediments under fish cages can not select for increased frequencies of resistance in the microflora. However, given the reduction in the biological activity of oxytetracycline that results from the concentrations of divalent cations in the marine environment (Lunestad and Goksøyr, 1990), it is possible that the oxytetracycline concentration of $4.6 \pm 3.7 \mu\text{g/g}$ found at farm A would only exert a very weak selection pressure. The concentrations of oxytetracycline detected by Samuelsen et al. (1992) would have exerted a much stronger selection pressure and may therefore have been causally related to the high resistance frequencies they detected. It should be noted, however, that even at these high concentrations, no simple correlation was observed between the amount of oxytetracycline in the sediment and the frequency of resistance. The initial therapy of the fish in two cages resulted in oxytetracycline concentrations of 285 and 189 ppm and resistance frequencies of over 100%. One of the cages was subjected to a second oxytetracycline therapy 51 days later. After this second treatment the oxytetracycline concentration under the re-treated cage had again risen to 287 ppm but the frequency of resistance in the sediments was approximately 25%. It is clear that more investigations will be required to identify all the factors leading to elevated resistance frequencies under fish cages.

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