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Residues of oxolinic acid in wild fauna following medication in fish farms

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ABSTRACT: Dispersion of oxolinic acid and occurrence of *Aeromonas salmonicida* in wild fauna were studied in animals captured in the vicinity of 2 aquaculture facilities during and after medication with this drug. Consumption of antibacterial agents in aquaculture has reached a considerable level. The major part of the drugs used reach the environment, either directly due to excessive feeding and reduced appetite of the cultured fish, or indirectly after having passed through the fish. Some of the drugs entering the environment are taken up by exploitable wild fish, shellfish and crustaceans, resulting in concentrations far above those accepted in food for human consumption in Norway. The concentration of oxolinic acid in muscle and liver was highest in pelagic fish like coalfish and mackerel. The mean concentration in muscle of all positive samples at the day of medication termination were 4.38 $\mu g\ g^{-1}$ and 0.42 $\mu g\ g^{-1}$ at 2 different farms. Maximum concentration of oxolinic acid in muscle samples was 12.51 $\mu g\ g^{-1}$. We found a simultaneous occurrence of oxolinic acid and the fish pathogenic bacterium *A. salmonicida* in the gut of both cultured and wild fish. This may lead to development and dispersion of resistant bacteria. Blue mussels *Mytilus edulis* harvested at one of the farms contained an elevated level of bacteria resistant to oxolinic acid.

INTRODUCTION

Bacterial diseases are considered a major problem in fish farming. Even though much work has been done to prevent outbreaks of such diseases, considerable amounts of antibacterial substances are still prescribed. In Norway, oxytetracycline (OTC), furazolidone (FZ) and trimethoprim/sulfa drugs were for many years the substances of first choice. However, since 1987 quinolones, especially oxolinic acid (OA), have found broader use (Fig. 1). In 1990 OA represented 74% by weight of all antimicrobial drugs prescribed in the treatment of fish in Norwegian aquaculture (statistics provided by Norwegian Medicinal Depot, Oslo).

Oxolinic acid (5-ethyl-5,8-dihydro-8-oxo-1,3-dioxolo{4,5-g}quinoline-7-carboxylic acid) is a weak acid with pK_a at 6.9. It is practically insoluble in acidic solutions but very soluble in alkaline media. In humans, OA is converted into at least 8 metabolites which are excreted principally as glucuronides (DiCarlo et al. 1968). Crew et al. (1971) examined the metabolism of OA in rats, rabbits and dogs; they reported that, quantitatively, none of these animals metabolised OA as humans. Qualitatively, however, rats, rabbits and dogs transformed oxolinic acid in a manner similar to that in humans. The molecular structure of oxolinic acid is shown in Fig. 2.

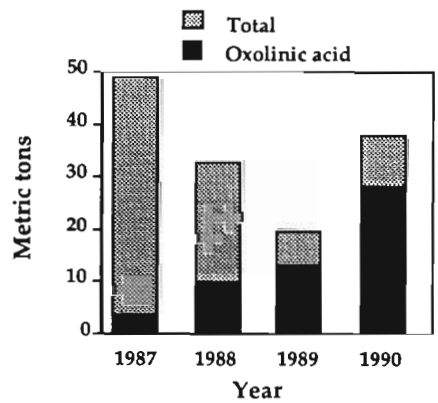

Fig. 1. Total amount of antibacterial agents and amount of oxolinic acid used in Norwegian aquaculture in the years 1987 to 1990

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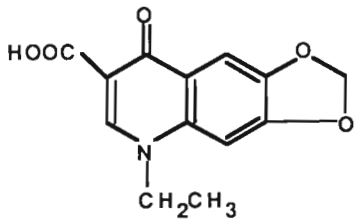

Fig. 2. Molecular structure of oxolinic acid

Bacterial infections in fish are commonly treated using food pellets containing the desired antibacterial substance. However, diseased fish often show reduced appetite and an over-feeding may occur, leading to medicated food pellets entering the surroundings. During feeding, fragments containing the antibacterial agent may fall off, or the drug may be released from the pellet in a dissolved form (Higuera et al. 1990). Further, preliminary experiments using OA as the antibacterial agent have shown that some of the drug eaten by the farmed fish is excreted in an unchanged active form via the urine or faeces (Samuelsen unpubl.). Hence, considerable amounts of the drug will enter the environment either directly due to overfeeding or indirectly after having passed through the animal under treatment. Therefore, during chemotherapy of farmed fish, the wild fauna may be exposed to varying amounts of drugs, sometimes on several occasions during a year

Particle-bound medicine can be taken up by filter-feeders such as appendicularians or mussels and dissolved drugs can be absorbed by organisms directly from the water (Nusbaum & Shotts 1981). Using a microbiological method, Møster (1986) found residues of OTC in blue mussels *Mytilus edulis* harvested 80 m from the application area. Medicated faeces and food pellets also reach the bottom deposits under the farms in considerable amounts (Jacobsen & Berglind 1988, Björklund et al. 1990, Samuelsen et al. in press). The occurrence of OTC in fish caught near a rearing facility for rainbow trout *Oncorhynchus mykiss* on the south-west coast of Finland was investigated by Björklund et al. (1990).

They found OTC levels ranging from 0.2 to 1.3 $\mu g\ g^{-1}$ in 8 muscle samples of bleak *Alburnus alburnus* L. collected on the last day of medication. The medication regime applied was 100 mg OTC $(kg\ fish)^{-1}\ d^{-1}$ for 10 d. They also found that roach *Rutilus rutilus* L., caught near a salmon farm 1 d after terminated medication, had OTC levels in muscle in concentrations close to the detection limit of the HPLC method (0.05 $\mu g\ g^{-1}$). The farms described by Björklund were located in an area with brackish water of salinity ca 5 ‰. Bleak and roach are typical species in brackish or freshwater fauna. In coastal areas of countries including Norway and Scotland, coalfish *Pollachius virens*, cod *Gadus morhua*, mackerel *Scomber scombrus*, ballan wrasse *Labrus bergylta* and haddock *Melanogrammus aeglefinus* are the most common species found in the vicinity of marine aquaculture sites (Carss 1990). No findings concerning possible OA residues in the wild fauna near Norwegian salmon farms have yet been published. The present study was undertaken to obtain information on the occurrence and concentrations of OA in the wild fauna in the vicinity of 2 salmon farms located on the west coast of Norway. The occurrence of the bacterium *Aeromonas salmonicida* in the gut content of both farmed and wild fish and the number of bacteria resistant to OA in *M. edulis* were also studied. *A. salmonicida* is the causative agent of furunculosis, at present the most severe disease of bacterial origin in Norwegian aquaculture.

MATERIALS AND METHODS

Sampling sites. Samples of cultivated fish, wild fish, crabs and blue mussels were collected at 2 marine aquaculture facilities on the west coast of Norway during October and November 1990. On Farm 1, 134 000 kg of salmon *Salmo salar* L. suffering from furunculosis were treated for 10 d using a total of 34 kg OA distributed in 11 200 kg moist pellets. This farm represents a typical Norwegian fish farm in size and stock. Average seawater temperature was 9 °C during the sampling period and the water depth under the cages varied between 40 and 70 m. The farm was located in a narrow strait with tidal currents in alternating directions.

On Farm 2, 18 000 kg salmon suffering from vibriosis (*Vibrio anguillarum*) were treated for 8 d using a total of 9.84 kg OA distributed in 1.928 kg dry pellets. Average seawater temperature was 11 °C during sampling and the water depth under the cages varied between 30 and 60 m.

Sampling. Samples were collected on the last day of medication (Day 0) at both farms and at Days 4, 7 and 13 (Farm 1) and Days 7 and 13 (Farm 2) following medication. Wild fish weighing between 0.6 and 5 kg were caught using fishing lines or with nets placed within 400 m of the farms. Crabs were caught both in nets and traps, and blue mussels were collected with a landing net or by divers. Samples of muscle, liver and blood were taken from the fish immediately after the catch was landed. Haemolymph and muscle samples were collected from the crabs. Haemolymph and blood samples were taken using a heparinized 1 ml syringe, centrifuged at 15 000 × *g* on the farm using a Biofuge A centrifuge (Heraeus Sepatech, Osterode am Harz, Germany) keeping only the plasma fraction. A

total of 46 samples of gut contents were taken from wild and cultivated fish at Farm 1 during the sampling period. All samples were kept on ice in the dark. Samples of the gut contents and blue mussels used in the microbiological assays were prepared and analysed on the day of sampling, at once on return to the laboratory. This was also the case with the plasma samples analysed for OA residues using high-performance liquid chromatography (HPLC). For determination of OA residues, the blue mussels, muscle samples from crabs and fish, and liver samples from fish were kept at –20 °C until analysed.

Analysis. Plasma samples (100 µl) were analysed for OA residues following the method described by Samuelsen (1990). Standard curves for OA in the range 0.01 to 1 and 1.0 to 2.5 µg ml^{-1} were prepared using drug-free plasma of salmon, coalfish and crab. Flumequine was used as internal standard in a concentration of 1.0 and 2.5 µg ml^{-1} plasma respectively.

The tissue samples were prepared for HPLC following the procedure of Samuelsen (1989) with minor modifications. Muscle (crabs and fish), liver (fish) and soft tissue homogenate (blue mussels) (1 g) from each organism were weighed, homogenized with two 20 ml portions of McIlvaine buffer (pH 3.6)-methanol (55-45) at high speed for 1 min and centrifuged for 10 min at 8000 × *g* in a Sorvall RC-5B refrigerated superspeed centrifuge (Du Pont Instruments, Newtown, CT, USA). Prior to the first homogenization 0.1, 1.0 or 5.0 µg flumequine dissolved in 0.1M NaOH was added as an internal standard. The amount of internal standard added to the muscle and liver samples were based upon the concentration of OA found in the corresponding plasma samples.

The combined supernatants were filtered (589 Black ribbon, Schleicher & Schuell, Dassel, Germany) and evaporated under reduced pressure at a temperature of 40 °C until 15 ml of the solution remained in the flask. Using 5 ml McIlvaine buffer (pH 3.6) for washing, the flask content was transferred to a separating funnel already containing 25 ml of dichloromethane. After gently shaking the separating funnel for ca 1 min, the 2 layers were allowed to separate and the dichloromethane fraction was transferred to a further separating funnel and rinsed with 10 ml of McIlvaine buffer (pH 3.6). The dichloromethane phase containing the drugs was transferred to a small beaker and evaporated to dryness in a water-bath (40 °C) under a stream of nitrogen. The residue was dissolved in 0.25 or 0.5 ml of 0.1M NaOH and centrifuged for 5 min at 15 000 × *g* (Biofuge A centrifuge). This solution (25 µl) was used for HPLC.

Standard curves for OA in the range of 0.01 to 0.1, 0.1 to 1.0 and 1.0 to 10 µg g^{-1} were prepared using drug-free samples of muscle (coalfish, crab and salmon), liver (coalfish, salmon) and homogenised soft tissue (blue mussel). Flumequine was used as internal standard in concentrations of 0.1, 1.0 and 5.0 µg g^{-1} tissue respectively. Standard curves were drawn by plotting the known drug concentration against the ratio of drug to internal standard peak height. These curves were also used to calculate the detection limits. The extraction recoveries of OA and flumequine in plasma and tissues were determined by comparing peak heights from the analysis of samples spiked with 1.0 µg g^{-1} OA and flumequine with peak heights resulting from direct injection of a standard. The chromatographic assay and HPLC equipment described by Samuelsen (1990) was used to analyse both the tissues and plasma samples.

Linear graphs (r = 0.97 to 0.99) were obtained for OA in plasma (0.01 to 2.5 µg ml^{-1}), homogenised soft tissue, muscle and liver (0.01 to 10 µg g^{-1}) samples. Detection limits were calculated to be 0.003 to 0.010 µg g^{-1} with a signal to noise ratio of 3, and recoveries were between 85.4 and 93.5 % when blank samples (1 g tissue, 100 µl plasma) were spiked with 1.0 µg OA. Lowest recoveries and highest detection limits were obtained for the liver samples. The standard curves for coalfish were used to calculate the OA residues in all species except salmon, crab and blue mussel.

The occurrence of OA in the gut contents of fish and the soft tissue of blue mussels was examined using a standard microbiological method applying an OA-sensitive organism (*Escherichia coli* B_6). Aliquots of 0.05 ml of the sample material was applied on a thin lawn of bacteria on Petri dishes using a sterile plastic syringe. The media used was Luriua Bertani Agar, a standard medium for cultivation of *E. coli* (Difco bacto agar 15 g, Difco bacto tryptone 10 g, Difco yeast extract 5 g, glucose 1 g, NaCl 10 g, distilled water to 1 l, pH adjusted to 7.4). Results were read after incubation at 37 °C for 12 h. Inhibition zones in bacterial growth indicated the presence of antibacterial substances in the sample material. When applying this method, identification of the agent in question cannot be done. However, it is not very likely that other substances either eaten or produced by the fish or produced by bacteria in the gut would give inhibition zones of the magnitude observed here. In this study we therefore consider growth inhibition of *E. coli* as a positive result for the occurrence of OA in the gut.

To quantify the number of OA resistant bacteria associated with the soft parts of the blue mussels a homogenate was plated on agar media containing 10 µg ml^{-1} OA. The number of colony forming bacteria on the media containing OA was compared to the number of bacteria growing on media without any antibacterial agent, indicating the percentage of bacteria resistant to OA in blue mussels. The media

Table 1 Residues of oxolinic acid in plasma, muscle and liver, total count of bacteria and the occurrence of *Aeromonas salmonicida* in faeces from wild fish and farmed salmon at Farm 1 Day: days following medication; Farm: samples taken from salmon inside the cages; 0, 50 and 100 m: distance from the farm; n: total number of samples examined. Coalfish = *Pollachius virens*, salmon = *Salmo salar*. Activity: number of samples containing faecal material with antibacterial activity; *A. salm.*: number of samples containing *A. salmonicida* in faeces; total count: total number of bacteria per ml faeces

Day	Location	n	Species	Mean concentrations in $\mu g\ g^{-1}$			Faeces		
				Muscle	Liver	Plasma	Activity	*A. salm.*	Total count
0	Farm	4	Salmon	0.7	0.4	0.08	1	4	3.0×10^9
0	0 m	8	Coalfish	5.99	7.66	1.50	8	0	7.4×10^9
4	Farm	5	Salmon	0.49	0.33	0.16	1	0	na
4	0 m	7	Coalfish	3.92	2.47	0.69	7	0	na
7	Farm	6	Salmon	0.06	0.04	0.06	0	2	2.7×10^8
7	0 m	1	Salmon	0.00	0.00	0.00	0	1	2.6×10^9
7	50 m	1	Salmon	0.23	0.47	0.18	1	1	1.2×10^9
7	100 m	1	Salmon	0.01	0.02	0.03	0	1	1.7×10^9
12	Farm	6	Salmon	0.00	0.01	0.00	0	2	1.1×10^8
12	0 m	7	Coalfish	0.03	0.02	0.00	0	1	2.8×10^9

na: not analysed

used was tryptone soya agar (TSA) in 70 % seawater as recommended by Lunestad & Goksøyr (1990) for determination of resistance of marine bacteria (tryptone soya broth 20 g, Difco bacto agar 15 g, 70 % seawater to 1 l). The plates were read after 3 d following aerobic incubation at 15 °C.

To determine the total number of bacteria and the number of *Aeromonas salmonicida* in the faeces of fish and blue mussel homogenate, each sample was homogenized for 1 min in a Waring blendor at low speed and diluted 1:1000 in prefiltered seawater. The samples were preserved with formaldehyde (final concentration 2 %) and the bacteria were stained using the techniques described by Hoff (1988) and Enger et al. (1989). These methods involve filtration of the sample through a 0.2 µm Nucleopore filter and a staining of the bacteria with 4,6-diamidino-2-phenylindole (DAPI). When applying this method, DAPI diffuses from an underlying supporting filter previously soaked in DAPI solution (100 $\mu g\ ml^{-1}$), staining the bacterial DNA. The bacteria present on the Nucleopore filter were also treated with monoclonal antibodies directed towards *A. salmonicida*. The antibodies used were diluted 1:800 in marine PBS (20 g NaCl and 4.3 g $MgCl_2 \cdot 6\ H_2O$ in 1000 ml distilled water adjusted to pH 7.2) containing 0.5 % bovine serum albumin (Sigma Chemical Company, St. Louis, MO, USA). After 50 min the filters carrying the bacteria were washed (3×) with aliquots of 3 ml marine PBS. One ml of commercial FITC-conjugated rabbit anti-mouse immunoglobulin (DAkO, diluted 1:500 in marine PBS) was added and the filters kept at room temperature for 30 min. The filters were washed twice with marine PBS before mounting on glass slides. The preparations were examined in a Zeiss standard fluorescence microscope with a HBO 50 W mercury lamp and a DAPI/FITC filter package as described by Enger et al. (1989). Immunofluorescent positive cells with a halo were only registered as positive counts of *A. salmonicida* if the bacteria also exhibited a positive DAPI signal.

RESULTS

A total of 46 wild fish and farmed salmon samples from Farm 1 were analysed for faecal antibacterial activity, total number of bacteria and the presence of *Aeromonas salmonicida* in faeces (Table 1). At Day 0, *A. salmonicida* was found in all of the 4 farmed salmon while none of the 8 coalfish tested were positive. Neither the farmed salmon nor the coalfish tested at Day 4 contained *A. salmonicida* in the gut. However, 7 d after termination of medication we found *A. salmonicida* in 2 out of 6 farmed salmon and in all 3 salmon caught at a distance of 0 to 100 m from the farm. On the 12th day, *A. salmonicida* was found both in the farmed salmon and in 1 out of 7 coalfish.

High concentrations of OA in plasma, liver and muscle were found at both Days 0 and 4 in pelagic species (coalfish and mackerel) as well as in demersal species (haddock and crabs). Liver samples from wild fish generally had higher residual OA concentrations at Day 0 compared to muscle samples, but this difference had leveled out by Day 4. OA concentrations, particularly in coalfish, were considerably higher than in the farmed salmon tested at Days 0 and 4 (Tables 2 & 3).

Fig. 3 shows the total catch of wild fish and crabs at Farms 1 and 2 and the number of OA-positive samples.

Table 2. Residues of oxolinic acid (μg g^{-1}) in wild fauna in the vicinity of Farm 1. n: total number of samples (in parentheses) and number of positive plasma, muscle, liver and homogenate samples. Mean: mean value of all positive samples; max: maximum concentration found. Coalfish = *Pollachius virens*, ballan wrasse = *Labrus berggylta*, ling = *Molva molva*, crab = *Cancer pagurus*, haddock = *Melanogrammus aeglefinus*, cod = *Gadus morhua*, pollack = *Pollachius pollachius*, salmon = *Salmo salar*, blue mussel = *Mytilus edulis*

Source of sample	Day 0			Day 4			Day 7			Day 13		
	n	Mean	Max	n	Mean	Max	n	Mean	Max	n	Mean	Max
Coalfish	(33)			(13)			(14)			(18)		
Plasma	33	1.56	7.12	13	0.99	3.11	10	0.16	0.65	2	0.03	0.04
Muscle	33	5.19	10.71	13	3.92	12.51	14	0.22	0.81	14	0.02	0.03
Liver	33	6.45	13.59	13	3.65	14.68	14	0.21	0.87	12	0.02	0.03
Ballan wrasse	(2)			(10)			(6)			(3)		
Plasma	2	0.01	0.19	6	0.04	0.15	3	0.04	0.07	0	–	–
Muscle	2	0.49	0.92	10	0.44	1.37	5	0.03	0.08	0	–	–
Liver	2	0.29	0.37	10	0.34	0.90	5	0.04	0.10	0	–	–
Ling	(3)			*			*			(1)		
Plasma	ns	–	–	–	–	–	–	–	–	–	–	–
Muscle	3	0.63	1.26	–	–	–	–	–	–	0	–	–
Liver	3	0.89	1.49	–	–	–	–	–	–	0	–	–
Haddock	(2)			(1)			*			(1)		
Plasma	ns	–	–	1	0.52	–	–	–	–	0	–	–
Muscle	2	5.02	8.99	1	1.88	–	–	–	–	1	0.01	0.01
Liver	2	5.21	9.21	1	0.72	–	–	–	–	1	0.01	0.01
Salmon	*			*			(3)			(2)		
Plasma	–	–	–	–	–	–	ns	–	–	ns	–	–
Muscle	–	–	–	–	–	–	2	0.12	0.23	1	0.1	0.1
Liver	–	–	–	–	–	–	2	0.30	0.47	1	0.06	0.06
Cod and pollack	(2)			(8)			(4)			(8)		
Plasma	2	0.05	0.09	7	0.03	0.05	4	0.03	0.06	0	–	–
Muscle	2	0.42	0.62	7	0.10	0.37	4	0.02	0.03	3	0.02	0.02
Liver	2	0.23	0.35	7	0.11	0.30	4	0.08	0.25	3	0.01	0.01
Blue mussels	(5)			(5)			(3)			–		
Homogenate	5	0.65	1.48	3	0.05	0.09	0	–	–	–	–	–
Crab	(5)			(13)			(10)			(12)		
Haemolymph	3	0.13	0.22	12	0.19	1.23	6	0.19	0.77	2	0.03	0.04
Muscle	5	0.81	3.77	11	0.45	1.61	6	0.08	0.25	4	0.03	0.09

* No catch; ns: no sample available

In this investigation a positive sample was defined as one having a residual OA concentration exceeding 10 ng g^{-1} tissue or 10 ng ml^{-1} plasma. Considering the large number of plasma samples on Day 7 at Farm 2 having no residual OA, we found it unnecessary to analyse the muscle and liver samples.

One sample (1 g) of homogenised tissue from 15 blue mussels collected on Farm 2 (Day 0) contained 0.07 μg g^{-1} of OA and had levels of OA-resistant bacteria more than 10 times higher than those found in similar samples from blue mussels collected 200 and 400 m away. The blue mussels collected at 200 and 400 m had no detectable OA residues. At Day 7, blue mussels collected at Farm 2 contained no OA and had levels of OA-resistant bacteria similar to the mussels from 200 and 400 m (Day 0).

DISCUSSION

Oxolinic acid (OA) was first described in 1968 (Kaminsky & Meltzer 1968). OA inhibits microbial growth by interfering with DNA gyrase activity and is effective on Gram-negative bacteria (Wolfson et al. 1989). Gram-positive bacteria and anaerobes are usually not susceptible to OA (Walter & Heilmeyer 1975, Wolfson et al. 1989). Single step mutations leading to high-level resistance towards OA in bacteria have been reported to occur with frequencies in the range 10^{-6} to 10^{-8}. This creates the possibility of emergence of resistant organisms during therapy (Wolfson et al. 1989). On the other hand there have been no descriptions of transferable or plasmid-mediated resistance towards OA (Wolfson et al. 1989).

Table 3. Residues of oxolinic acid (µg g^{-1}) in wild fauna in the vicinity of Farm 2. n: total number of samples (in parentheses) and number of positive plasma, muscle and liver samples. Mean: mean value of all positive samples; max: maximum concentration found. Coalfish = *Pollachius virens*, crab = *Cancer pagurus*, cod = *Gadus morhua*, haddock = *Melanogrammus aeglefinus*, pollack = *Pollachius pollachius*, salmon = *Salmo salar*, flounder = *Platichthys flesus*, mackerel = *Scomber scombrus*

Source of sample	Day 0			Day 7			Day 13		
	n	Mean	Max	n	Mean	Max	n	Mean	Max
Coalfish	(4)			(5)					
Plasma	4	0.96	2.41	1	0.02	0.02	–	–	–
Muscle	4	1.04	2.42	1	0.02	0.02	–	–	–
Liver	4	1.86	5.60	1	0.01	0.01	–	–	–
Mackerel	(6)			(2)					
Plasma	5	0.16	0.73	0	–	–	–	–	–
Muscle	5	0.37	2.23	na	–	–	–	–	–
Liver	5	0.43	2.04	na	–	–	–	–	–
Cod	(2)			(2)					
Plasma	0	–	–	0	–	–	–	–	–
Muscle	2	0.03	0.05	na	–	–	–	–	–
Liver	2	0.02	0.03	na	–	–	–	–	–
Pollack	(2)			(16)					
Plasma	2	0.03	0.03	0	–	–	–	–	–
Muscle	2	0.03	0.03	na	–	–	–	–	–
Liver	2	0.03	0.04	na	–	–	–	–	–
Salmon	(2)			*					
Plasma	0	–	–	–	–	–	–	–	–
Muscle	0	–	–	–	–	–	–	–	–
Liver	0	–	–	–	–	–	–	–	–
Flounder	(3)			(4)					
Plasma	1	0.10	0.10	0	–	–	–	–	–
Muscle	1	0.03	0.03	na	–	–	–	–	–
Liver	1	0.01	0.01	na	–	–	–	–	–
Crab	(4)			(22)			(11)		
Haemolymph	2	0.02	0.02	8	0.09	0.56	0	–	–
Muscle	2	0.01	0.02	8	0.13	0.87	1	0.02	0.02

* No catch; na = not analysed

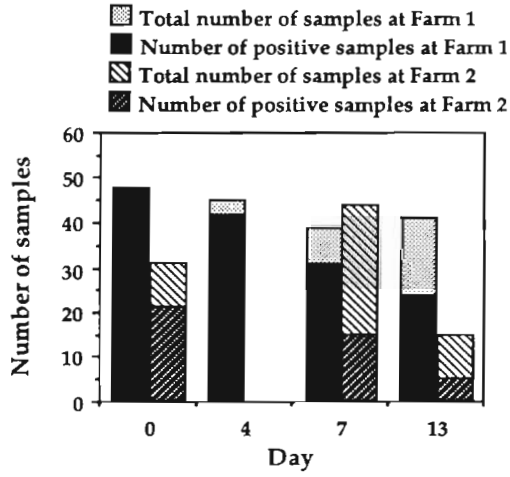

Fig. 3. Total number of samples and number of positive muscle samples at Farms 1 and 2. Number of positive samples at Farm 2, Day 7, are based on results from the plasma analysis

High doses of OA in fish have been reported to cause haematological disturbances and adverse effects on liver (Miyazaki et al. 1984).

In higher animals, OA is associated with toxic effects on the central nervous system (CNS) and with gastrointestinal irritation (Goldberg et al. 1961, Walter & Heilmeyer 1975, Mandel & Sande 1985, Crumplin 1988). Furthermore, OA is suspected to interfere with gene transcription in mammalian cells (Crumplin 1988). Due to the relatively high incidence of adverse effects and the narrow spectrum of activity, OA has never been considered a first line antimicrobial agent in clinical practice. However, the newer quinolones are promising. The incidence of adverse effects with the third generation quinolones seems to be reduced and the spectrum of activity has been broadened, making the new compounds more attractive for the treatment of bacterial diseases in humans (Moellering 1989).

In this investigation we examined a total of 225 fish, 76 crabs and 30 samples of blue mussels. Residues of

OA were detected in coalfish, mackerel, cod, haddock, whiting, ling, pollack, ballan wrasse, wild salmon, crabs and blue mussels. On the day medication was terminated the average concentration of OA in positive muscle samples of wild fish was 4.38 $\mu g\ g^{-1}$ at Farm 1 and 0.42 $\mu g\ g^{-1}$ at Farm 2. The highest concentration of OA in muscle (12.51 $\mu g\ g^{-1}$) was found in a coalfish caught at Farm 1 on Day 0. Even in fish caught 400 m away from Farm 1 at a depth of nearly 100 m, residues were found. These findings exceed by far the drug concentrations allowable in food for human consumption in many countries, including Norway. These countries have general regulations requiring the complete absence of drug residues in food. Thus, in practice, the detection limit of the method employed is then the maximum level permitted. At present the detection limit of the microbiological test in use in Norway is 0.10 $\mu g\ g^{-1}$ [H. Evensen (Directorate of Fisheries, Control laboratory) pers. comm.]. The detection limits of the different HPLC methods available are 0.0005 to 0.010 $\mu g\ g^{-1}$ (Bjørklund 1989, Rogstad et al. 1989, Andresen & Rasmussen 1990, Steffenak et al. 1991). Twelve days after termination of medication, only minor concentrations of OA could be found in the tissues of the species examined. The farmed salmon contained much lower concentrations of OA than most of the wild living fishes. This reflects the usually low appetite of diseased fish.

A considerable number of the fish examined had high concentrations of OA in the gut at Day 0 and 4. It is not yet known whether the high antibacterial activity is due to residues of drug from food intake or to excreted OA and microbiologically cleaved OA-conjugates from the bile. However, HPLC analysis of samples collected from salmon treated with OA showed residues of both unchanged and conjugated OA in the bile (Samuelsen unpubl.). It is possible for drug conjugates in the bile to be transformed to active drug by the intestinal bacteria and then reabsorbed (enterohepatic circulation). Four other fish farms were situated in the same area as Farm 1. In these farms, a total of 660 tonnes of salmon were treated with OA during the same period as treatment at Farm 1. This may partly account for the high residues of OA in the fish and large number of positive samples at Farm 1.

When OA was detected in the intestinal contents, the inhibition zones produced varied between 12 and 30 mm in diameter. Fish containing OA in faeces usually had high concentrations of the drug in liver, muscle and plasma. Considering the positive correlation with the HPLC method, the microbiological assay could be valuable in the investigation of drug residues in both farmed and wild organisms since the microbiological method is rapid and does not require expensive equipment.

Several authors reported an increase in the number of resistant faecal bacteria during antibacterial treatment in humans (Goldberg et al. 1961, Datta et al. 1971). This has also been reported to occur in the treatment of fish (Aoki et al. 1972, Toranzo et al. 1984, Schlotfeldt et al. 1985). In previous years an increase in the occurrence of the pathogenic fish bacteria *Aeromonas salmonicida* and *Yersinia ruckeri* (causal agent of enteric redmouth disease) resistant to OA has been reported (Hastings & McKay 1987, O'Grady et al. 1987, Tsoumas et al. 1989). It is therefore reasonable to believe that this decrease in susceptibility is caused by the widespread use of OA in the treatment. On the other hand Sugita et al. (1989) reported that OA did not markedly influence the number of bacteria, the bacterial ability to resist OA and the intestinal bacterial flora of goldfish *Carassius auratus* during a standard treatment.

Aeromonas salmonicida and OA were present simultaneously in the gut of wild and farmed salmon (Table 1). This may permit *A. salmonicida* to develop resistance towards OA and, via faeces, be spread to the environment. In this manner furunculosis can be spread to the wild fish population and further to other fish farms by OA resistant bacteria.

Marine bivalves have frequently been employed in the assessment of environmental pollution. Their wide distribution and low level of xenobiotic metabolism combined with a sessile adult behavior and high filter activity makes these organisms especially suitable for the detection of pollutants (Krieger et al. 1981). In our investigation we found low to moderate concentrations of OA in the blue mussels examined. Despite low concentrations of OA in blue mussels at Farm 2, an increased percentage of OA-resistant bacteria could be observed in samples taken immediately after termination of medication. One explanation may be that the mussels have filtered out resistant bacteria shed from the intestine of fish receiving the drug. At low pumping rates blue mussels have been reported to be capable of retaining particles as small as 2 µm. At high pumping rates all particles larger than 6 µm are retained (Dral 1967, Vahl 1972). However, most marine bacteria are too small to be retained efficiently by blue mussels if not aggregated or present on debris (Wright et al. 1982). Some of the bacteria in the intestine of fish may well be associated with faecal particles which are filterable by blue mussels. Bacteria found on soft tissue surfaces of blue mussels at Day 7 after termination of medication had no elevated level of bacteria resistant to OA. There is reason to believe that the bacterial flora of blue mussels reflects the flora present in the water. When the concentration of OA in the farmed and wild fish intestine decreases, the number of resistant bacteria released into the seawater will also decrease.

Since it is a well established principle that residues of medicines given to livestock animals should not be found in food for human consumption, considerable efforts are made to prevent drug transfer in this way. However, the present investigation clearly shows that during medication the wild fauna near a fish farm is exposed to the drug in use. If wild fish, crustaceans or mussels are harvested in the vicinity of an aquaculture facility during medication, the risk of drugs reaching consumers is high. In the case of OA, the drug is also rather thermostable and no reduction in antibacterial activity could be observed when an aqueous solution of the drug was boiled for 15 min to simulate conditions resembling normal treatment of fish.

Combined with a compulsory announcement of medication, collection of medicated food particles that fall through the net pens and optimisation of the medication strategy will reduce the problem. Since there is no obvious reason to distinguish between free-living and farmed animals in regard to drug residues, monitoring of exploitable wild animals exposed to drugs from aquaculture, similar to that with farmed fish, should be considered.

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