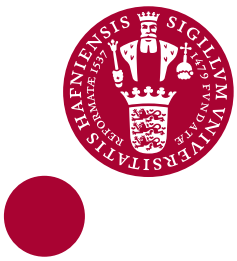

Antimicrobial resistance in aquatic environments

with emphasis on the fish pathogen Flavobacterium psychrophium and oxytetracycline resistance

Bruun, M. S.

Publication date:
2001

Document version
Publisher's PDF, also known as Version of record

ANTIMICROBIAL RESISTANCE IN AQUATIC ENVIRONMENTS

- With emphasis on

the fish pathogen *Flavobacterium psychrophilum*

and oxytetracycline resistance

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PhD thesis

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2001

ISBN 87-988517-0-5

This thesis submitted for the degree of Doctor of Philosophy is based on five scientific publications and a literature review

Results! Why, man, I have gotten a lot of results.
I know several thousand things that won't work.
- *Thomas Edison*

Til min familie

Summary

The overall purpose of this PhD-thesis was to improve the general knowledge of antimicrobial resistance in bacteria related to and inhabiting the aquatic environment. Phenotypic resistance, the genetic background and transferability of the resistance were studied. The aquatic habitat investigated was confined to the freshwater environment in relation to aquaculture in a major Danish stream.

The occurrence, persistence and spread of antimicrobial resistance among bacteria associated with four Danish rainbow trout farms located at the same stream was investigated. Water, sediment and fish were sampled monthly during a 15-months period. Antimicrobial agents of different classes all used in Danish aquaculture were included (oxolinic acid, sulfadiazin/trimethoprim, amoxicillin, florfenicol and oxytetracycline). The resistance levels at the inlet, in a pond and at the outlet of each farm were determined as the fraction of resistant culturable bacteria to each of the antimicrobial agents included. In addition, all the major fish pathogenic bacteria to rainbow trout reared in fresh water in Denmark as well as an environmental indicator bacterium were included to give a more differentiated impression of the resistance. The fish pathogens were *Flavobacterium psychrophilum* the etiological agent of bacterial cold water disease also known as rainbow trout fry syndrome, *Yersinia ruckeri* the cause of enteric redmouth disease and *Aeromonas salmonicida* subspecies *salmonicida* causing furunculosis. *Aeromonas hydrophila* is an omnipresent bacterium in aquatic environments and was included as an indicator organism. This group was later expanded to include all motile *Aeromonas* species.

This PhD study focuses mainly on *F. psychrophilum* and its resistance to antimicrobial agents and also oxytetracycline resistance in general in the examined aquatic environments and spread of this resistance by conjugal transfer.

As *F. psychrophilum* is a fastidious bacterium requiring specific growth conditions it was necessary to develop a reliable method for susceptibility testing of this bacterium. The method, described in Paper 1, was developed and subsequently used to test the susceptibility towards the included antimicrobial agents of a comprehensive collection of previously isolated *F. psychrophilum* strains and isolates collected during this project. Characterization of the resistance patterns showed that resistance was widespread towards oxytetracycline, oxolinic acid and sulfadiazine/trimethoprim. Amoxicillin resistance was absent among the early isolates but occurred widespread in the later years. All investigated isolates were susceptible to florfenicol.

Paper 2 comprises the results of the fish farm sampling and subsequent analysis of antimicrobial resistance. A significant effect of fish farms on the occurrence of resistance was shown. The number of culturable bacteria resistant to oxytetracycline was higher in the water samples from the pond and the outlet than in the inlet samples. In addition, higher numbers of oxytetracycline resistant *F. psychrophilum* and motile *Aeromonas* spp. were demonstrated at the outlet. A similar pattern was detected for the motile *Aeromonas* spp. regarding resistance to sulfadiazine/trimethoprim and oxolinic acid. This effect was apparently localised to the single fish farm environment, as no accumulation of resistant bacteria was observed further down the stream.

During the successive characterization of the nature of the resistance (Paper 3 + 4) all isolates were examined for their plasmid content and bacteria resistant to oxytetracycline were screened for specific tetracycline determinants (A-E) by using a multiplex PCR method. In contrast to earlier reports (comprising mainly clinical isolates) only 30 % of the oxytetracycline resistance found in motile *Aeromonas* spp. were assigned to one of the tested determinant classes and only two of 63 oxytetracycline resistant *F. psychrophilum* were classified (Tet C). No resistant *Y. ruckeri* isolates were detected among the 134 identified isolates and no *A. salmonicida* was isolated during the sampling period.

Bacteria resistant to sulfadiazine/trimethoprim were examined for class 1 integrons that always carries sulfonamid resistance and in addition have the ability to integrate other resistance genes as gene cassettes. No integrons were detected in *F. psychrophilum* but the occurrence of integrons could account for the detected resistance to sulfonamides in the motile *Aeromonas* spp. The involved gene cassettes were sequenced and shown to contain genes coding for resistance to trimethoprim, aminoglycosides and chloramphenicol.

In vitro experiments to determine transfer of oxytetracycline resistance from motile *Aeromonas* spp. with large plasmids yielded 17 out of 40 successful conjugal transfers to *Escherichia coli*. Transfer was also shown between motile *Aeromonas* spp. and to *Y. ruckeri* but no transfer was seen to *F. psychrophilum*. Transfer of R-plasmids was demonstrated in situ. Membrane filter chamber submersed into the stream from which the isolates originated were used to mimic the natural conditions.

Finally treatment efficiency of fish experimentally infected with *F. psychrophilum* strains having different susceptibilities to oxytetracycline were assessed (Paper 5). This study stresses the importance of testing bacterial susceptibility on a suitable growth medium facing an outbreak of rainbow trout fry syndrome.

Sammendrag

Det overordnede formål med denne ph.d.-afhandling var at udvide og underbygge den eksisterende viden om resistens-forholdene overfor udvalgte antimikrobielle stoffer hos bakterier i et akvatisk miljø. Fænotypisk resistens, det genetiske grundlag for resistensen og eventuel overførbarhed skulle belyses. Projektet blev afgrænset til ferskvandsmiljøer i relation til dambrug ved et større dansk åløb.

Forekomst, persistens og spredning af antibiotikaresistente bakterier blev undersøgt. Bakterierne blev isoleret ved fire danske dambrug beliggende langs den samme å og alle med regnbueørredopdræt. Vandprøver, sediment og fisk blev indsamlet månedligt gennem 15 måneder og efterfølgende blev prøverne viderebehandlet i laboratoriet og bakteriefloraen blev underkastet nærmere resistensbestemmelser. Undersøgelsen inkluderede antibiotika som tilhører forskellige klasser men alle er blevet anvendt i danske dambrug. Det drejer sig om oxolinsyre, sulfadiazin/trimethoprim, amoxicillin, florfenicol og oxytetracyklin. For hvert antibiotikum blev resistens-niveauet ved indløbet, i en dam og ved udløbet fra hver af de fire dambrug bestemt som andelen af resistente dyrkbare bakterier i forhold til den totale dyrkbare bakterieflora. For at opnå et mere detaljeret billede af resistensforholdene blev det desuden besluttet at inddrage de vigtigste sygdomsfremkaldende bakterier hos regnbueørreder i danske dambrug samt en indikator-bakterie. De udvalgte fiskepatogene bakterier var *Flavobacterium psychrophilum*, som forårsager yngeldødeligheds-syndromet eller bacterial cold water disease, *Yersinia ruckeri*, der er årsag til rødmundsyge og furunkulose-bakterien *Aeromonas salmonicida* subspecies *salmonicida*. *Aeromonas hydrophila* blev udvalgt til at være indikatorbakterie, da den findes udbredt i akvatiske miljøer – senere modificeret til at omfatte alle bevægelige *Aeromonas* arter.

Denne ph.d.-afhandling fokuserer først og fremmest på den fiskepatogene bakterie *F. psychrophilum* og dennes resistensforhold samt på resistens overfor oxytetracyklin generelt i de undersøgte akvatiske miljøer og overførsel af denne resistens.

Da *F. psychrophilum* er en langtsomt-voksende bakterie med specielle vækstkrav bestod første del af projektet i at udvikle en pålidelig metode til bestemmelse af bakteriens følsomhed overfor de udvalgte antimikrobielle stoffer. Denne metode blev efterfølgende anvendt dels på en større laboratorie-kollektion af *F. psychrophilum* og dels på stammer isoleret i forbindelse med dette projekt. Metoden samt resistensforholdene er beskrevet i Paper 1, og det ses, at udbredt resistens var tilstede overfor oxytetracyklin, oxolinsyre og sulfadiazin/trimethoprim. De tidlige isolater var stort set følsomme overfor amoxicillin mens resistens var ret udbredt hos bakterier isoleret de seneste år. Alle de testede isolater var følsomme overfor det nyligt introducerede florfenicol.

Efter prøveindsamlinger og efterfølgende analyser af resistensforholdene i relation til dambrug blev resultaterne sammenskrevet i Paper 2, som bl.a. påviser en signifikant effekt på forekomsten af resistente bakterier i relation til dambrugene. Indholdet af oxytetracyklin-resistente dyrkbare bakterier var højere i vand fra damme og udløb end i indløbsvand og der blev også fundet markant flere oxytetracyklin-resistente *F. psychrophilum* samt bevægelige *Aeromonas* spp. i udløbsvandet. For de bevægelige *Aeromonas* spp. var samme tendens desuden signifikant for sulfadiazin/trimethoprim og oxolinsyre. Effekten var tilsyneladende lokal, da der ikke blev observeret en akkumulering af resistente bakterier nedstrøms i åløbet.

I det videre arbejde med karakterisering af især den fundne resistens (Paper 3 + 4) blev alle isolater undersøgt for indhold af plasmider og oxytetracyklinresistente bakterier blev screenet vha.

en multiplex PCR metode for de hyppigst forekommende tetracyklin resistens determinanter (A-E) ifølge den eksisterende litteratur. I modsætning til tidligere studier (af overvejende kliniske isolater) kunne kun 30 % af oxytetracyklin resistensen hos de bevægelige *Aeromonas* spp. henføres til de nævnte resistens determinant klasser og kun to ud af 63 resistente *F. psychrophilum* kunne klassificeres (Tet C). Der blev i øvrigt ikke fundet nogle resistente *Y. ruckeri* blandt de 134 identificerede isolater, ligesom der slet ikke blev isoleret furunkulosebakterier i nogen af dambrugene i hele indsamlingsperioden.

Sulfadiazin/trimethoprim resistente bakterier blev undersøgt for indhold af klasse 1 integroner, som altid koder for sulfonamidresistens og desuden kan inkorporere yderligere resistensgener i form af genkassetter. Integroner blev ikke fundet hos *F. psychrophilum*, mens forekomst af integroner kunne forklare den observerede sulfonamidresistens hos de bevægelige *Aeromonas* spp. Sekventering af de involverede genkassetter viste, at de kodede for resistens overfor trimethoprim (dihydrofolat-reduktase gener), aminoglycosider og kloramfenikol.

Laboratorieforsøg med overførsel af oxytetracyklinresistens fra bevægelige *Aeromonas* spp. med store plasmider viste, at 17 ud af 40 isolater kunne overføre resistensen til *Escherichia coli* ved konjugation. Desuden kunne overførsel påvises mellem bevægelige *Aeromonas* spp. og til *Y. ruckeri*, men ikke til *F. psychrophilum*. Spredning af resistensplasmider blev også påvist under tilnærmede naturlige forhold i dialysekamre ved udløbet af et dambrug.

For at belyse forholdene omkring behandlingseffekt og resistens blev regnbueørredyngel inficeret med tre *F. psychrophilum* stammer med forskellig følsomhed overfor oxytetracyklin, hvorefter fiskene blev medicineret via foderet og effekten af behandlingen blev fulgt (Paper 5). Vigtigheden af at teste følsomheden overfor relevante antibiotika i hvert enkelt tilfælde af sygdom understreges.

Table of contents

Accompanying papers

Paper 1 Bruun, M.S., Schmidt, A.S., Madsen, L., Dalsgaard, I. 2000. Antimicrobial resistance patterns in Danish isolates of *Flavobacterium psychrophilum*. Aquaculture. 187, 201-212.

Paper 2 Schmidt, A.S., Bruun, M.S., Dalsgaard, I., Pedersen, K., Larsen, J.L. 2000. Occurrence of Antimicrobial Resistance in Fish-Pathogenic and Environmental Bacteria Associated with Four Danish Rainbow Trout Farms. Applied and Environmental Microbiology. 66, 4908-4915.

Paper 3 Schmidt, A.S., Bruun, M.S., Dalsgaard, I., Larsen, J.L. 2001. Incidence, distribution and spread of tetracycline resistance determinants and integron encoded antibiotic resistance genes among motile aeromonads from a fishfarming environment. Submitted

Paper 4 Bruun, M.S., Schmidt, A.S., Dalsgaard, I., Larsen, J.L. 2001. Conjugal transfer of oxytetracycline resistance in vitro and in membrane filter chambers: transfer between environmental aeromonads, fish pathogenic bacteria and *Escherichia coli*. Submitted

Paper 5 Bruun, M.S., Madsen, L., Dalsgaard, I. 2001. Efficiency of oxytetracycline treatment in Rainbow Trout experimentally infected with *Flavobacterium psychrophilum* strains having different in vitro susceptibilities. Submitted

Introduction

Aquaculture has been an expanding industry worldwide during the last decades. The largest growth has been in the Far East especially China, but inland aquaculture production has also been increasing in the European Community from 154000 tons in 1984 to 251000 tons in 1996 (FAO 1998). The Danish freshwater aquaculture production almost doubled during the eighties but has been rather constant during the last decade with 33445 tonnes produced in 1998 (Sortkjær et al. 2000). As in any other animal production high concentration of animals predisposes to disease and as a consequence treatment of the diseased animals is required. Antimicrobial agents are used to combat various bacterial diseases in production animals as well as in humans, pet animals and crops.

Infections caused by bacteria resistant to multiple antimicrobial agents have been a major concern and constraint especially in hospitals during the last two decades (Davies 1994). Indiscriminate use of antimicrobial agents in society and hospitals has been blamed, but also the use in animal production and subsequent transfer of resistant bacteria or resistance genes to human pathogens is suspected to compromise the clinical efficacy of some broad-spectrum antimicrobial agents (DuPont and Steele 1987, Levy 2000) though the current evidence is mainly circumstantial. Likewise untreatable infections occur in veterinary medicine, but are rare and do not yet represent a significant problem.

Attention has been paid to the use of antimicrobial agents in aquaculture and the possibilities of resistance development in fish pathogenic bacteria and the indigenous bacteria exposed to these agents (Michel 1986, Smith et al. 1994). The focus on use of antimicrobial agents in aquaculture is due to the fact that administered drugs to some extend will end up in a more or less active form in the aquatic environment (Halling-Sørensen et al. 1998). The impact of the inevitable drug residues on the freshwater environmental bacteria is sparsely dealt with in literature (Spanggaard et al. 1993, Guardabassi et al. 1998, Guardabassi et al. 2000a) though some reports on fish pathogenic bacteria, mainly in sea water, describe increased levels of resistance to the applied antimicrobial agents (de Grandis and Stevenson 1985, Inglis and Richards 1991). Resistance genes are often located on transferable genetic elements as plasmids or transposons (Aoki 1988, Aoki 1997) and this enables the acquired resistance to spread. Transfer of resistance genes to other fish pathogenic bacteria, to the omnipresent bacterial community and ultimately to bacteria causing disease in humans should be thoroughly investigated to obtain a reliable risk assessment.

The work presented in this PhD-thesis is to some extend derived from a larger project and is a part of The Danish Veterinary Environmental Research Program (s. Veterinær miljøforskning) initiated by The Ministry of Agriculture and Fisheries in 1997. The main objective of the programme was to monitor and investigate antimicrobial resistance in production animals and the environment in Denmark. Other parts of the project dealt with bacterial survival strategies in the environment, resistance in relation to pig manure and soil, and spread of resistance in soil, whereas the subproject named “Monitorering af forekomst, spredning og persistens af patogener og antibiotikaresistens, specielt i relation til fiskeopdræt og vandmiljøer” aimed for rainbow trout production and the surrounding freshwater environment.

Aim of the study

The overall purpose of this PhD-thesis was to improve the general knowledge of antimicrobial resistance in bacteria related to and inhabiting the aquatic environment.
The objectives as stated in the PhD application have been:

1. To examine the occurrence of tetracycline resistance and tetracycline resistance genes in fish pathogenic bacteria.
2. To examine the effect of treatment towards certain fish pathogenic bacteria in vitro
3. To examine the dissemination/transfer of certain resistance genes in vitro
4. To examine the transfer and persistence of resistance genes in the environment

Three lines, more or less intertwined, were to be followed:

1. Freshwater environment in relation to fish farms.
   - All sampling and thus isolates originating from this study were from four freshwater fish farms situated at Vejle Å, a major stream in Jutland, Denmark. Samples were collected during a 15 months period. Water and sediment were sampled as well as several fish from each farm.
2. The fish pathogen *Flavobacterium psychrophilum*
   - The culturable fraction of the environmental bacteria was examined in terms of the proportion of bacteria resistant to selected antimicrobial agents. But also specific bacterial species were isolated, identified and further examined. This thesis will focus on *F. psychrophilum* and to a certain extent the indigenous group of bacteria referred to as motile *Aeromonas* spp. In addition selective media were used to isolate *Yersinia ruckeri* and *Aeromonas salmonicida*, which will only be sparsely dealt with.
3. The antimicrobial agent oxytetracycline
   - The selection of antimicrobial agents to be used in the project was based on the agents used in Danish freshwater fish farms and a desire to include antimicrobial agents from different groups. Oxytetracycline resistance was assessed in detail, as this resistance is known to be located on plasmids or transposons and thus be able to spread horizontally in and between bacterial populations.

Chapter 1

Background and the rationale of the experiments performed

This chapter provides background information on the choices made in relation to materials used and methods employed during the present study. It offers knowledge of the thoughts and planning preceding the experiments presented in the accompanying papers as well as the underlying principles of these experiments and work that were not included in the papers.

1.1 Initial considerations

Selection of fish farms was based on three main issues. The number of farms to include was limited to four, which seemed manageable in respect to workload. Secondly, they should be located at the same stream, to facilitate detection of differences in resistance levels through the system. Finally the uppermost and the last farm employing stream water were selected as a "control" and a farm exposed to water from all other farms at the stream, respectively. A schematic representation of the location of the fish farms and a table containing information about the respective farms can be found in Paper 2.

Water, sediment and fish were included as sampling materials. This represented three different habitats, and variations in bacterial numbers, species composition and resistance patterns would probably exist. To assess the effect of the individual fish farm, water and sediment samples were obtained before each farm (inlet), from a pond, and at the outlet of the farms. Two apparently healthy fish were taken from a pond and if disease was present diseased fish were sampled as well.

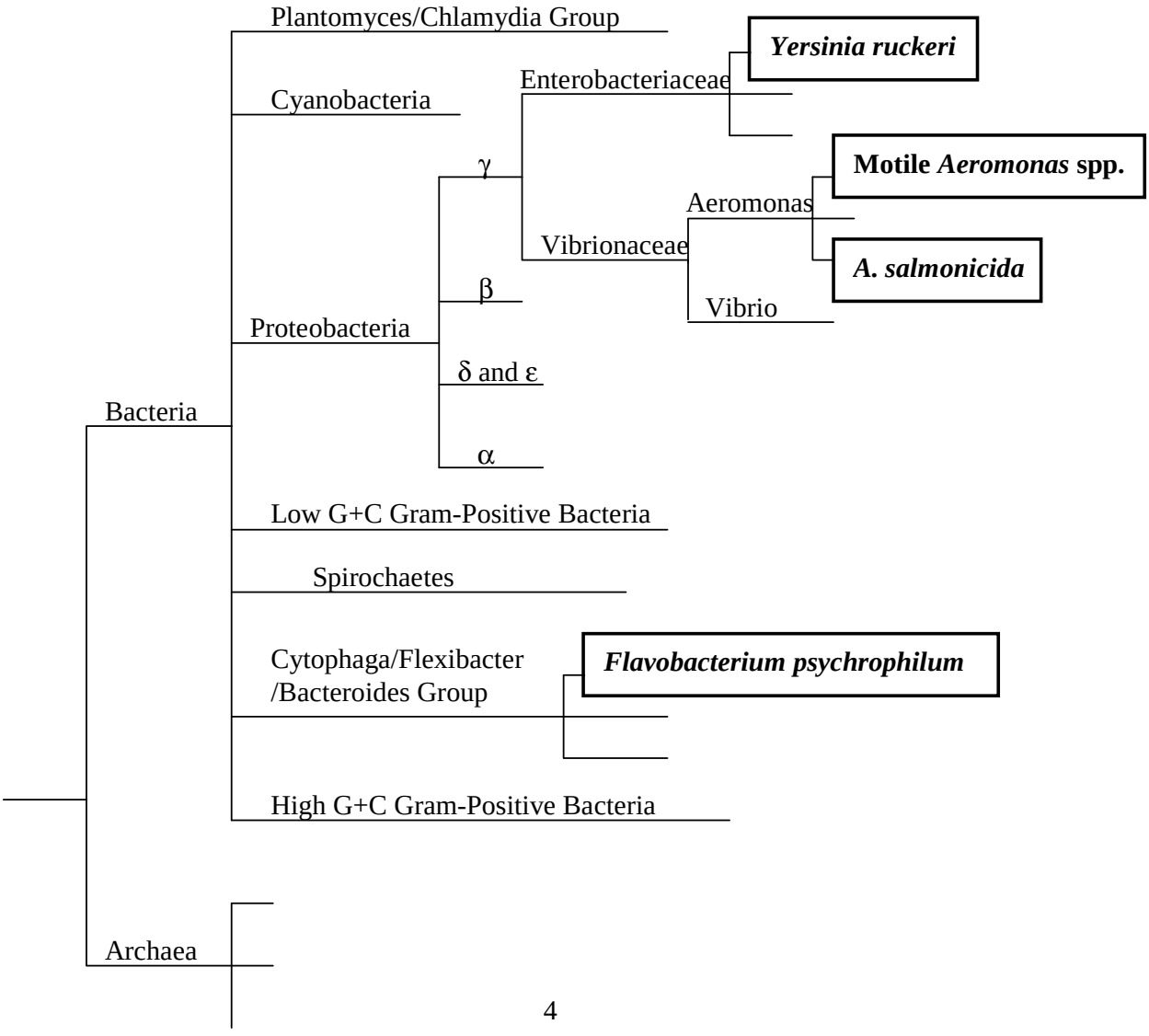
As one of the objectives was to monitor the development of resistance levels, a measure of the "total resistance" was needed. Several conventional and genetic methods were available but we settled for differential plating - a conventional method where the same sample was plated on a non-selective medium and plates with the same basic medium added fixed levels of the selected antimicrobial agents. This makes it possible to calculate the fraction of resistant culturable bacteria to each of these antimicrobial agents.

In addition, antimicrobial resistance in various relevant species was to be assessed. All the major freshwater fish pathogenic bacteria to rainbow trout in Denmark (Dalsgaard and Madsen 2000) as well as an environmental indicator bacterium were included. The fish pathogens were *Flavobacterium psychrophilum* causing rainbow trout fry syndrome (RTFS), *Yersinia ruckeri* – the cause of enteric redmouth disease (ERM) and *Aeromonas salmonicida* causing furunculosis. *Aeromonas hydrophila* was included as an indicator organism, as literature stated that this species is an ubiquitous bacterium in natural aquatic environments (Noterdaeme et al. 1991) and in addition often carrying antimicrobial resistance and R-plasmids (Andersen and Sandaa 1994, Miranda and Castillo 1998, Goñi-Urriza et al. 2000). Due to various problems in identification of the species *A. hydrophila* from environmental samples this was later changed to comprise all the motile *Aeromonas* spp. The phylogenetic classification of the included bacteria can be seen in Figure 1.

Using conventional methods to assess antimicrobial resistance require isolation and/or culture of the microorganism in question. In contrast, genetic methods as PCR amplification of specific resistance gene sequences followed by sequencing of the obtained product or PCR-RFLP and different hybridisation techniques as recently reviewed (Cockerill III 1999) or introduction of potential recipients and subsequent isolation of the known bacterium that have picked up

resistance genes (Sørensen et al. 1996), require only isolation or transfer of the genetic material coding for antimicrobial resistance and not isolation of the implicated bacteria. Using the conventional methods gives the possibility of obtaining the original resistant bacterium to use in further investigations, but this also means that a large proportion of bacteria are excluded from the investigation, as they are non-culturable. It has been estimated that only 0.1 to 5% of the environmental bacterial community are culturable (Staley and Konopka 1985, Amann et al. 1995). The reason for this could be that a large fraction of the population is dormant or non-active or it could mean that only a small fraction of the bacteria is alive (Zweifel and Hagström 1995).
Selection of antimicrobial agents was based on the use in Danish freshwater fish farms. Both antimicrobial agents currently in use and agents used during the last decades were considered. Currently, only oxolinic acid (OXA), and sulfadiazin (SDZ) and trimethoprim (TMP) in combination (S/T) formulated as food premixes are licensed for use in aquaculture in Denmark. As these two antimicrobial formulations are effective against ERM and furunculosis but not RTFS it is possible to obtain a permit to use amoxicillin (AMX), florfenicol (FLO) or previously oxytetracycline (OTC) to treat RTFS outbreaks.

Fig. 1 Schematical phenogram based on maximum parsimony analysis of small subunit ribosomal DNA sequence
(adapted from http://lsvl.la.asu.edu/mic494/html/phylogenetic_tree.html)



1.2 Sample collecting and processing.

Water samples were collected in sterile 100 ml plastic flasks. Two samples were taken at each sample site approximately 10 cm beneath the water surface. Sediment was sampled with a bottom collector (van Veen type), and two samples of the 1 cm top layer were transferred aseptically to plastic flasks. All samples were cooled to 5˚C and processed later the same day. Back in the laboratory ten-fold dilutions were prepared from the water samples and 100 µl were plated from 10^{-2} and 10^{-1} on plain Tryptone Yeast Extract Salts (TYES) agar (Holt et al. 1993) and from 10^{-1} and 10^{0} on the TYES plates containing antimicrobial agents. Due to higher bacterial numbers ten-fold dilutions of sediment samples were made to 10^{-6} and subsequently plated from 10^{-6} to 10^{-4} on TYES agar and from 10^{-4} and 10^{-3} on the antimicrobial containing TYES plates. In addition to the plain and antimicrobial containing TYES agar plates, spread plating was done on blood agar (BA) mainly to obtain haemolytic bacteria (e.g. *Aeromonas* spp.), ribose-ornithin-dextrin agar (ROD) a selective medium for *Y. ruckeri* isolation (Furones et al. 1993), coomassie brilliant blue agar (CBB) (Cipriano et al. 1992) to detect *A. salmonicida* and finally pril-ampicillin-dextrin-ethanol agar (PADE) for isolation of motile *Aeromonas* spp. (Imziln et al. 1997).

From apparently healthy fish the second gill arch from each side were excised and whirl-mixed in 10 ml physiological saline (0.9 % NaCl). From this, tenfold dilutions were prepared and plated on all the previously mentioned media. Likewise, a skin area of one times two centimetres was scraped using a sterile scalpel and the mucus layer was transferred to 10 ml PS, whirl-mixed, diluted and plated (Fig. 2). If disease was present at the time of sampling, two to four diseased fish were brought back to the laboratory for necropsy and inoculation on BA, TYES, PADE and ROD agar from spleen, kidney, brain and lesions if any were present.

Furthermore, isolates of fish pathogens were received from fish-practitioning veterinarians either in the form of diseased fish, a culturette swab or as more or less pure culture from internal organs on appropriate agar media.

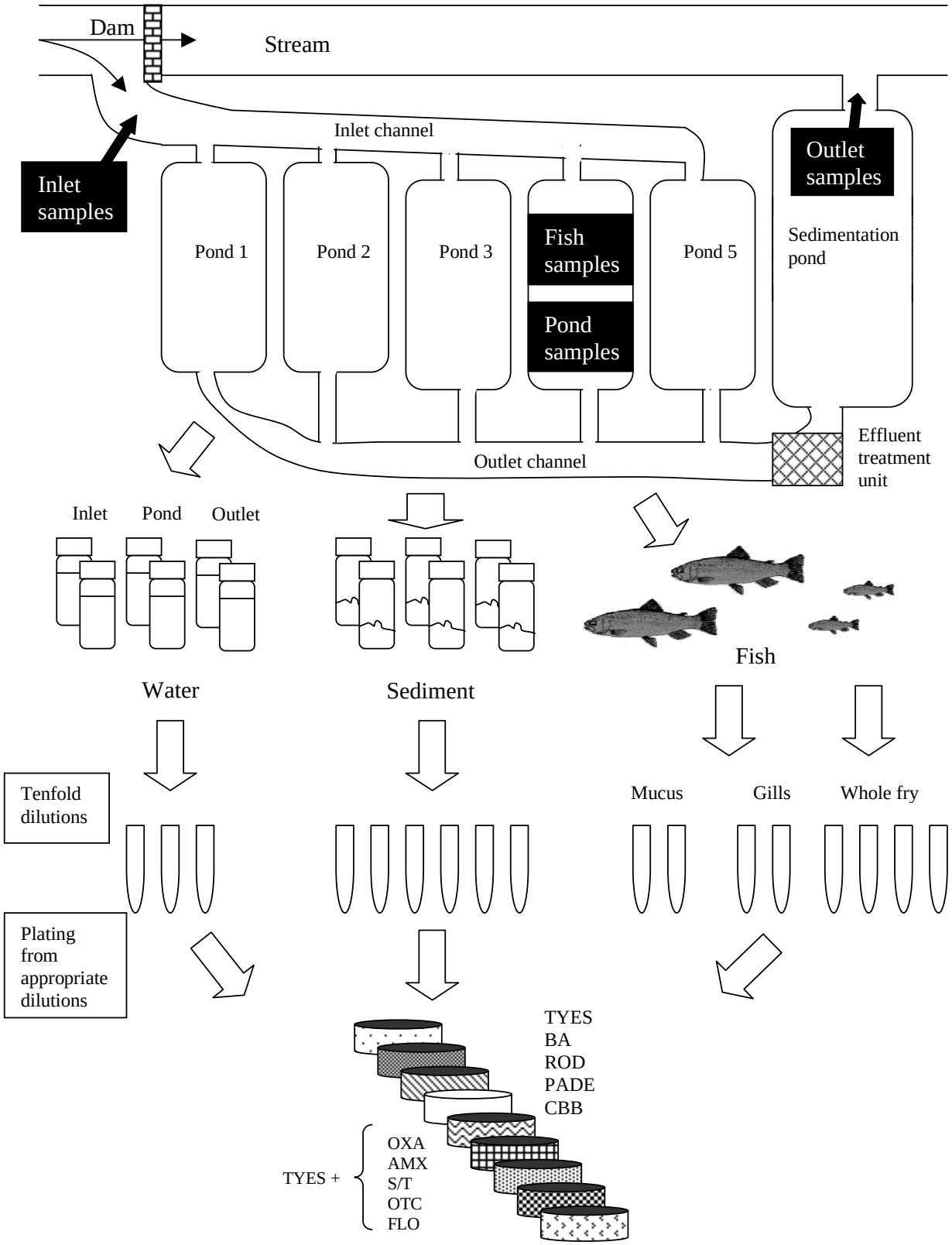
1.3 Bacterial identification

As mentioned two approaches were taken – one was an initial identification of selected fish pathogens and *Aeromonas hydrophila* to species level and subsequent investigation of these bacteria. Isolation and identification procedures of these bacteria are described in details for each species in Chapter 2. Much work was initially done to identify *A. hydrophila* to species level, but in the end this was abandoned and the isolates were instead grouped together as motile mesophilic *Aeromonas* spp. These were later separated into several groups (Paper 3).

The other parallel approach was a method to determine the resistant fraction of the culturable bacteria. TYES agar was used as it supported growth of *F. psychrophilum*, was used routinely in the laboratory and supposedly would yield a higher number of culturable bacteria than other more nutrient rich media. Selective plates contained the following concentrations of the selected antimicrobial agents: 10 µg OTC/ml, 50 µg SDZ/ml in combination with 10 µg TMP/ml and 4 µg/ml of OXA, AMX and FLO, respectively.

The idea was to pick a wide variety of the culturable environmental bacteria from both TYES-agar and selective plates and identify them to species level or at least perform numerical taxonomy based on several biochemical reactions and subsequently group the bacteria. It soon appeared impossible to identify the bacteria to species level and after doing some rather laborious biochemical typing and placing the bacteria into broad groups this approach was abandoned as well. From numerical taxonomy of 172 isolates, 18 groups or clusters were found. The criteria

Fig 2. Schematic presentation of a freshwater fish farm and the sampling sites



TYES = Tryptone Yeast Extract Salts agar. OXA = oxolinic acid (4 µg/ml), AMX = amoxicillin (4 µg/ml) S/T = sulfadiazin/trimethoprim (50 µg SDZ/ml in combination with 10 µg TMP/ml), OTC = oxytetracycline (10 µg/ml), FLO = florfenicol (4 µg/ml), BA = blood agar, ROD = ribose-ornithin-dextrin agar, PADE = pril-ampicillin-dextrin-ethanol agar, CBB = coomassie brilliant blue agar.

used were morphology, Gram-staining, motility, catalase, cytochrome oxidase and reaction in Hugh & Leifson (oxidative and/or fermentative). The initial idea was to assure or confirm that the species composition remained stable from time to time, as this could affect the resistance pattern of the bacterial community sampled. The information obtained did not match the work put into the numerical taxonomy of such complex bacterial communities existing in natural environments and it was not possible to confirm that different samples contained comparable bacterial communities from these rather crude groupings. This led to the conclusion that working with bacteria isolated from environmental sources was much more complicated than working with bacteria of clinical origin and the fraction of resistant culturable bacteria were to be used regardless of possible variations in species composition.

1.4 Susceptibility testing

Bacterial susceptibility towards antimicrobial agents differs in respect to the species in question and also intraspecies variations can occur as a result of acquired resistance. A dilution method is the most quantitative way to test antimicrobial susceptibility as it yields the minimum inhibitory concentration (MIC) directly (Thornsberry 1991). Other methods like disk diffusion or E-test rely on diffusion of the antimicrobial agent through the agar and this means that growth rate/incubation time is crucial for interpretation of the results (Thornsberry 1991).

As *F. psychrophilum* is a fastidious bacterium and requires a nutrient-poor medium, low temperature and extended incubation time the existing veterinary guidelines for susceptibility testing (NCCLS 1997) had to be modified as described in Paper 1. It was possible to use the control strains proposed by NCCLS even at 15˚C incubation temperature. The strains used were *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27835, *Staphylococcus aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212. The use of control strains differ from a recently proposed method of susceptibility testing of bacteria associated with fish diseases. This method uses the NCCLS guidelines as blueprint as well and is comparable to the method described here but uses only one fish pathogenic bacterium as control strain on each plate (Alderman and Smith 2001). Even if I do not agree in the use of only one control strain, the draft is a step in the right direction of international standardisation for future susceptibility testing (Alderman and Smith 2001), and several important considerations have been discussed in the follow-up paper by Smith (2001).

1.5 Plasmids

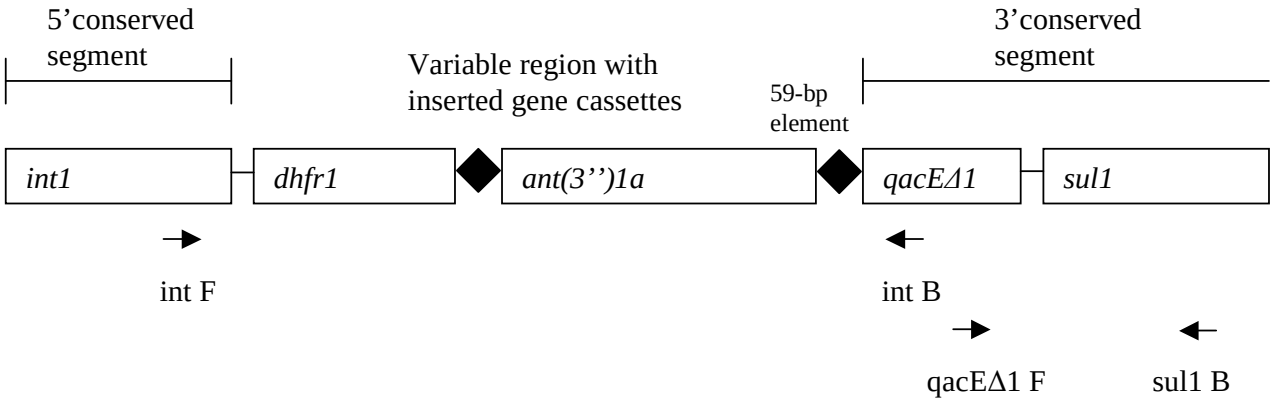
The initial step in assessing the genetic background of the detected resistance was screening for plasmids. Plasmids are circular extrachromosomal self-replicating genetic elements often carrying resistance genes. Plasmids are often implicated in acquired resistance as a means of horizontal transfer within or between bacterial species. The information obtained was suggested to link certain resistances to specific plasmid sizes, or detection of large plasmids could indicate transferable plasmids and through that a potential to transfer resistance.

Many reports have shown that plasmids often are implicated in occurrence and spread of antimicrobial resistance in general and in aquatic environments (Toranzo et al. 1984, de Grandis and Stevenson 1985, Hedges et al. 1985, Baya et al. 1986, Aoki 1988, Ansary et al. 1992, Chaudhury et al. 1996, Aoki 1997, Adams et al. 1998, Chandrasekaran et al. 1998, Dröge et al. 2000).

1.6 Integrons

As many isolates showed resistance towards S/T and a relatively recent discovered genetic element (the integron) always carries resistance to sulfonamides often in addition to one or more integrated resistance genes (Hall and Stokes 1993) it was decided to screen for these integrons (Fig. 3)

Fig. 3 Structure of a class 1 integron and primer annealing sites (Hall and Stokes 1993, Schmidt et al. 2001).



Several reports deal with integrons, gene cassettes and their implication in spread of resistance genes (Hall and Stokes 1993, Hall and Collis 1995, Recchia and Hall 1995, Jones et al. 1997, Sandvang et al. 1997, Martinez-Freijo et al. 1998, Mazel et al. 2000) also specifically in aquatic environments (Rosser and Young 1999, Petersen et al. 2000, Schmidt et al. 2001, Paper 2). For the initial screening it was decided to use primers int F and int B (Sandvang et al. 1997) (Fig. 3). This approach had the advantage that the inserted gene cassette was amplified along with small parts of the conserved elements. The size of the amplicon depended on the size of the gene cassette that subsequently could be sequenced for detection and identification of possible resistance genes or cut by restriction enzymes (Paper 3).
Primers qacEΔ1 F and sul1 B were used to confirm the presence of integrons and to detect integrons lacking the *sul1* gene as seen in other investigations (Rosser and Young 1999).

1.7 Tetracycline resistance determinants

Resistance to oxytetracycline was relatively common among motile aeromonads and *F. psychrophilum* and as the aim of the project specifically included tetracycline resistance it was decided to screen for tetracycline resistance determinants. At that time 30 tetracycline resistance determinants had been described (Table 1) but a closer look revealed that only a few determinants accounted for a vast majority of the tetracycline resistance detected in Gram-negative bacteria in general (Lee et al. 1993, Roberts 1996, Poulsen 1999, Schnabel and Jones 1999) and from aquatic environments in particular (DePaola et al. 1993, Andersen and Sandaa 1994, Guardabassi et al. 2000b). Consequently, the five determinants Tet A, Tet B, Tet C, Tet D and Tet E were selected for further investigations.

Table 1 Classification of known tetracycline resistance genes

Class	Localisation	Mechanism	Distribution	GenBank accession no.
A	plasmid/transposon	efflux	Gram-negative: *Aeromonas, Citrobacter, Edwardsiella, Escherichia, Klebsiella, Plesiomonas, Proteus, Pseudomonas, Salmonella, Serratia, Shigella, Vibrio*	X00006
B	transposon	efflux	Gram-negative: *Actinobacillus, Aeromonas, Citrobacter, Enterobacter, Escherichia, Haemophilus, Klebsiella, Moraxella, Pasteurella, Plesiomonas, Proteus, Providencia, Salmonella, Serratia, Shigella, Treponema, Yersinia, Vibrio*	J01830
C	plasmid	efflux	Gram-negative: *Citrobacter, Enterobacter, Escherichia, Proteus, Pseudomonas, Salmonella, Serratia, Shigella, Vibrio*	J01749
D	plasmid/chromosome	efflux	Gram-negative: *Aeromonas, Citrobacter, Edwardsiella, Enterobacter, Escherichia, Klebsiella, Pasteurella, Plesiomonas, Salmonella, Shigella, Vibrio, Yersinia*	X65876
E	plasmid	efflux	Gram-negative: *Aeromonas, Alcaligenes, Escherichia, Providencia, Pseudomonas, Serratia, Shigella, Vibrio*	L06940
F	transposon	efflux	*Bacteroides*	Unsequenced
G	plasmid/integron	efflux	Gram-negative: *Pseudomonas, Salmonella, Vibrio*	S52437
H	plasmid/transposon	efflux	*Pasteurella multocida*	U00792
I	?	efflux?	*Enterobacteriaceae*	Unsequenced
J	chromosome	efflux	*Proteus mirabilis*	AF038993
K	plasmid	efflux	Gram-positive: *Bacillus, Clostridium, Enterococcus, Eubacterium, Listeria, Mycobacterium, Nocardia, Peptostreptococcus, Staphylococcus, Streptococcus, Streptomyces*	M16217
L	plasmid/chromosome	efflux	Gram-positive: *Actinomyces, Bacillus, Clostridium, Enterococcus, Eubacterium, Listeria, Mycobacterium, Peptostreptococcus, Staphylococcus, Streptococcus, Streptomyces*	M11036/ X08034
M	conjugative transposon/plasmid/ chromosome	ribosomal protection	Gram-positive: *Actinomyces, Aerococcus, Bacterionema, Bifidobacterium, Clostridium, Corynebacterium, Enterococcus, Eubacterium, Gardnerella, Gemella, Listeria, Mycoplasma, Peptostreptococcus, Staphylococcus, Streptococcus, Ureaplasma* Gram-negative: *Campylobacter, Eikenella, Fusobacterium, Haemophilus, Kingella, Neisseria, Pasteurella, Prevotella, Veillonella*	X04388
O	plasmid/chromosome	rib. protection	Gram-positive: *Aerococcus, Enterococcus, Lactobacillus, Mobiluncus, Peptostreptococcus, Staphylococcus, Streptococcus,* Gram-negatives: *Campylobacter*	M18896
P(A)	plasmid	efflux	*Clostridium, Helicobacter*	L20800
P(B)	plasmid	rib. protection	*Clostridium*	L20800
Q	conjugative transposon/ plasmid	rib. protection	Gram-positive: *Eubacterium, Lactobacillus, Mobiluncus, Peptostreptococcus, Streptococcus* Gram-negative: *Bacteroides, Capnocytophaga, Mitsuokella, Porphyromonas, Prevotella, Veillonella*	X58717
S	plasmid	rib. protection	Gram-positive: *Enterococcus, Lactococcus, Listeria*	L09756
T	chromosome	rib. protection	*Streptococcus*	L42544
U	plasmid	rib. protection	*Enterococcus*	U01917
V	chromosome	efflux	*Mycobacterium smegmatis*	AF030344
W	transposon	rib. protection	*Butyrivibrio*	AJ222769
X	conjugative transposon	enzymatic	*Bacteroides*	M37699
Y	?	efflux	*Escherichia coli*	Not available
Z	plasmid	efflux	*Corynebacterium*	AF121000
30	chromosome	efflux	*Agrobacterium*	AF090987
31	?	efflux	*Aeromonas*	Not available
OtrA	chromosome	rib. protection	Gram-positive: *Streptomyces, Mycobacterium*	X53401
OtrB	chromosome	efflux	*Streptomyces rimosus*	AF079900
OtrC	chromosome	unknown	*Streptomyces*	Unsequenced
Tcr3	chromosome	efflux	*Streptomyces aureofaciens*	D38215
Tet	chromosome	rib. protection	*Streptomyces lividans*	M74049

Table reworked using (Chopra and Roberts 2001), (Levy et al. 1999) and the references cited herein combined with searches in GenBank nucleotide database via Entrez at http://www.ncbi.nlm.nih.gov/ and information available at the Belgian Biosafety server at http://biosafety.ihe.be/AR/ARmenu.html .

PCR primer sets specific for each determinant were described by Hansen et al. (1996). As the PCR products obtained from these primer sets were of similar sizes, some new primers were designed to be able to perform multiplex PCR i.e. to test for multiple determinants in the same PCR reaction. The method was first described by Guardabassi et al. (2000b). Examples of screening by multiplex PCR are shown in Fig. 4 and Fig. 5.

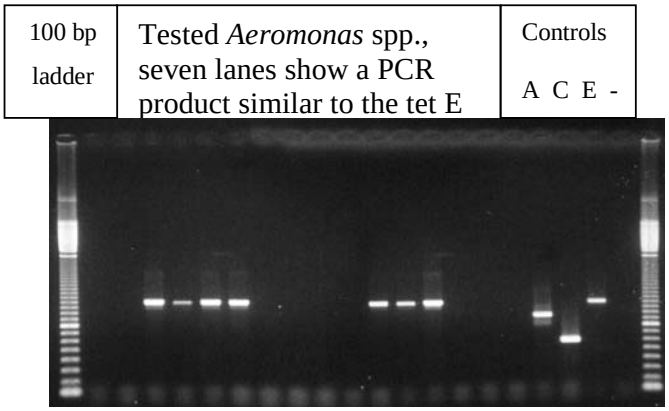


Fig. 4
Example of a multiplex PCR screening of tetracycline determinants A,C and E

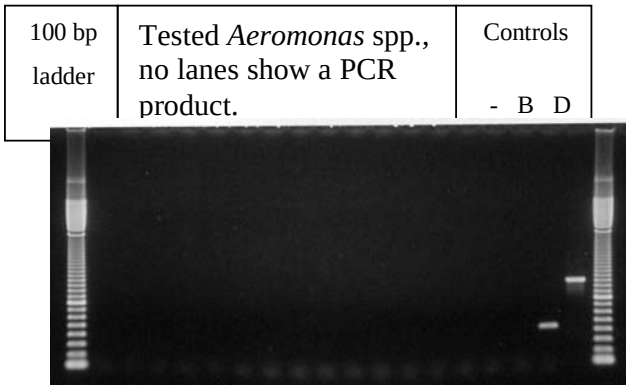


Fig. 5
Example of a multiplex PCR screening of tetracycline determinants B and D

1.8 Transfer experiments

In order to examine the potential of the resistant bacteria to transfer their resistance genes to other bacteria, various transfer experiments were performed. The spread of resistance genes in bacterial populations will be described in details in part 3.4 of chapter 3, and only the motive of the experiments will be discussed here. Again, oxytetracycline resistance was chosen, as tetracycline resistance determinants often are located on mobile elements. As conjugation was the mechanism to be studied, potential donor bacteria were selected. Selecting oxytetracycline resistant bacteria with large plasmids limited the donor bacteria to the group of motile *Aeromonas* spp. as no *F. psychrophilum* isolates harboured any large plasmids and all *Y. ruckeri* isolates were susceptible to oxytetracycline. A total of forty isolates complied with the two criteria, and a stepwise approach was taken to investigate the transfer potential under different conditions. First step was to screen all forty isolates in vitro by filter mating (Fig. 6) under what was believed to be optimum conditions with high bacterial load, extensive surface area and nutrient rich environment at 20°C (Genthner et al. 1988). To mimic natural conditions, the next set-up consisted of sterile water and sediment in a test tube. Afterwards transfer experiments were carried out using membrane filter chambers (McFeters and Stuart 1972) both in a laboratory aquarium containing tap water and finally, in the stream from which the isolates originated (Fig. 7).

1.9 Treatment trial

To demonstrate the usefulness of the obtained in vitro susceptibility data (Paper 1) treatment trials were performed. The in vivo trials use a previously introduced experimental infection model (Madsen and Dalsgaard 1999) and subsequently a medication with OTC via the feed. The infection model was based on intraperitoneal injection of *F. psychrophilum* and treatment was started the day after infection using 100 mg oxytetracycline/kg fish for ten days. Deaths were recorded daily and dead fish were examined. Tissue from spleen, kidney and brain were incubated on TYES agar at 15 °C for up to 10 days for microbiological confirmation of RTFS.
The purpose was to examine the effect of OTC treatment on rainbow trout fry with RTFS caused by strains of *F. psychrophilum* with different MIC values. No similar studies have apparently been performed on fish infected with *F. psychrophilum* or other fish pathogens.

Fig. 6 Filter mating flow diagram

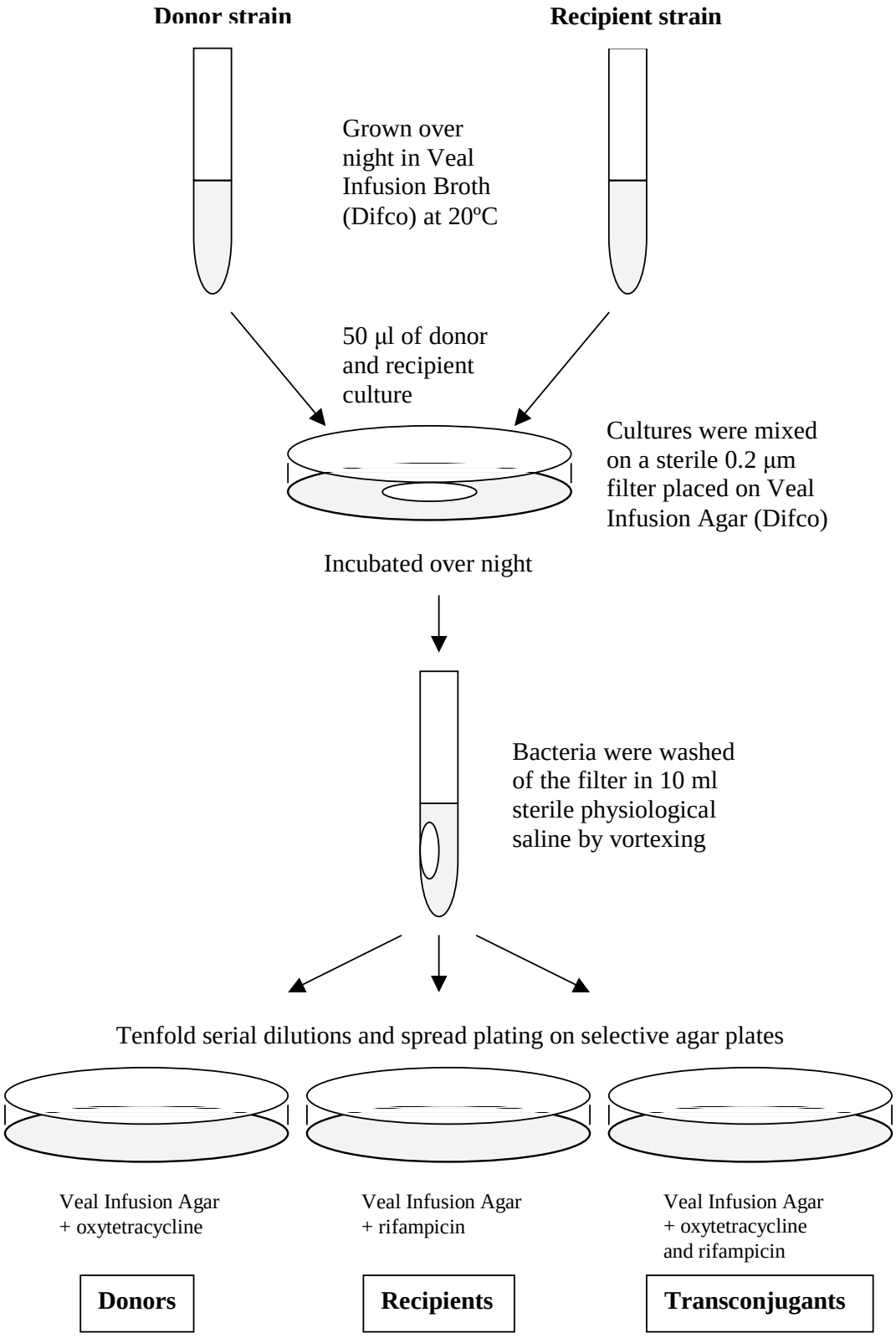
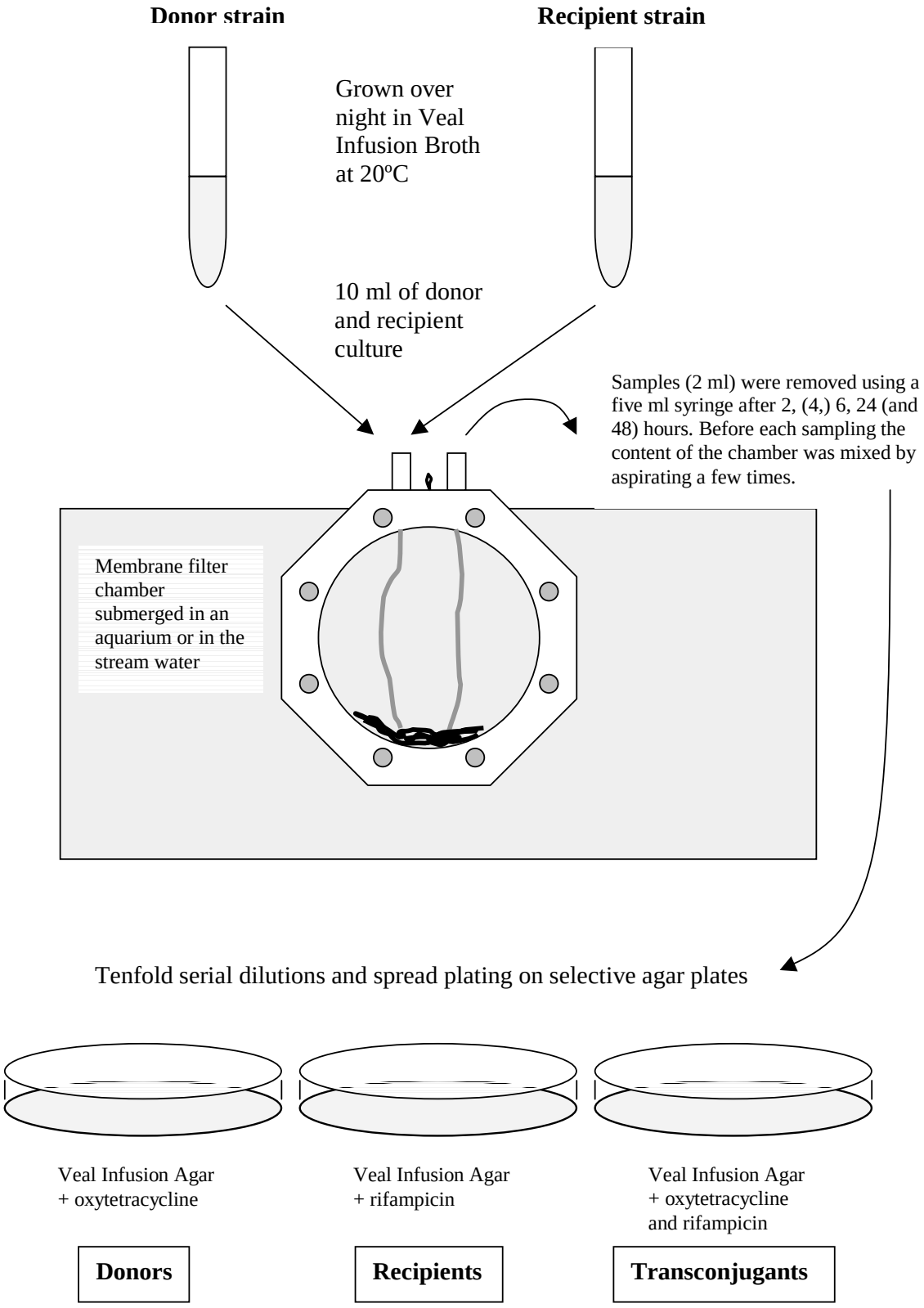


Fig. 7 Flow diagram of a mating experiment using the membrane filter chamber assay



Chapter 2

The bacteria

2.1 Culturable bacteria

The bacterial diversity in natural environments is high and using differential plating only the culturable bacteria and their resistance phenotypes were being investigated. It can be discussed whether this fraction is representative of the total bacterial community but it can be used to detect differences in resistance levels as described in Paper 2. Increased number of OTC resistant culturable bacteria was detected at the fish farm outlets. These results are strengthened by similar findings for individual species and determining resistance levels within particular species is in my opinion a superior method. Differential plating is labour consuming and a large data-material is necessary if subtle effects shall be proven.

2.2 *Flavobacterium psychrophilum*

Flavobacterium psychrophilum (Bernardet et al. 1996) is the causative agent of bacterial cold water disease (BCWD) seen primarily in salmonids. BCWD was first described in 1946 in USA (Borg 1948), and is now enzootic in several countries (Holt et al. 1993, Shotts and Starliper 1999). In Europe the disease in fry is called rainbow trout fry syndrome (RTFS), and has for 15 years caused serious problems in rainbow trout (*Oncorhynchus mykiss* Walbaum) hatcheries (Bernardet et al. 1988, Bruno 1992, Lorenzen et al. 1991, Toranzo and Barja 1993, Wiklund et al. 1994, Sarti and Giorgettii 1996). In Denmark it has become one of the main causes of losses in rainbow trout fry and fingerlings (Lorenzen 1994, Dalsgaard and Madsen 2000).

The species *F. psychrophilum* has undergone some taxonomic changes (Bader and Shotts 1998) since Borg (1948) introduced it as *Cytophaga psychrophila* due to its gliding motility and low growth temperature. The taxon was transferred to genus *Flexibacter* (Bernardet and Grimont 1989) and finally Bernardet et al. (1996) moved it to its current position in genus *Flavobacterium*. It has thus been placed in three different genera all belonging to the phylogenetic branch or phylum known as the *Cytophaga-Flexibacter-Bacteroides*-group (Fig. 1). So it is not included in the proteobacteria phylum as most Gram-negative bacteria.

2.2.1 Isolation and identification

After each sampling *F. psychrophilum*-like yellow colonies were picked from TYES plates with or without antibiotics. Diseased fish yielded pure culture from one or more internal organs but as a diversity of yellow colonies were detected from the environmental samples presumptive *F. psychrophilum* isolates were picked mainly from plates containing S/T. S/T added to TYES was used as a selective medium as a vast majority of *F. psychrophilum* isolates are resistant to this combination of antimicrobial agents, although the type strain (NCMB1947) was found to be less resistant than other investigated isolates. Further biochemical characterisation previously described by Pacha (1968) was used to identify the species. Presumptive *F. psychrophilum* formed yellow-pigmented colonies on TYES agar at 15˚C. They were catalase positive and cytochrome oxidase negative, filamentous Gram-negative rods with no growth on BA or at 37˚C and not capable of degrading carbohydrates. Confirmation was done by serology (Madsen 2000) and a species-specific PCR method (Toyama et al. 1994, Wiklund et al. 2000).

2.2.2 Susceptibility testing

Antimicrobial resistance in *F. psychrophilum* is insufficiently examined. Only few authors have addressed the subject (Lorenzen 1994, Wiklund et al. 1994, Rangdale et al. 1997, Dalsgaard and Madsen 2000). Paper 1 introduces a standard method for determination of minimum inhibitory concentration (MIC) in *F. psychrophilum.* Existing veterinary guidelines from The National Committee for Clinical Laboratory Standards (NCCLS 1997) was used with modifications in medium composition and incubation conditions. The medium used was Müller-Hinton agar as proposed by NCCLS, but modified according to Hawke and Thune (1992) to comply with the specific growth demands of *F. psychrophilum* (MDMH agar plates). Examples of two plates containing different concentrations of OTC are shown in Figures 8 and 9.

Resistance was widespread towards OTC (68%), OXA (66%) and S/T (98%) (Paper 1). In addition, occurrence of OTC resistance was significantly higher at the outlet compared to the inlet (Paper 2) indicating that factors in the fish farm promote development or transfer of resistance to OTC in *F. psychrophilum*. The causative factors might be selective pressure from antimicrobial agents, a favourable environment for gene transfers in the form of biofilm or large particular surfaces e.g. in sediment, in the surface mucus layer or in the gut of the fish or due to a high load of organic materials – this has yet to be clarified, but this study showed no simple correlation between use of antimicrobial agents and resistance levels (Paper 2). AMX resistance was absent among the isolates from 1994 and 1995 but occurred widespread in the years 1997 (38%) and 1998 (36%) (Paper 1). No resistance to florfenicol was detected but it has been seen in other bacteria (Kim and Aoki 1996, Arcangioli et al. 1999) and might develop in the long term or be acquired by *F. psychrophilum* from other fish pathogens or environmental bacteria. This process will probably be enhanced if bacteria are subjected to selective pressure.

The results presented in Paper 5 show that it is valid to predict whether or not OTC will be efficient in treating RTFS from in vitro data of susceptibility of the isolate responsible for the outbreak in question and the pharmacokinetic data of OTC in rainbow trout. This will most likely also be the case for other antimicrobial agents, fish species and bacterial pathogens as well, but it remains to be assessed. The importance of doing susceptibility testing when facing an outbreak at a fish farm is obvious, but as shown the selection of media for antimicrobial susceptibility testing of *F. psychrophilum* is important as also pointed out by Dalsgaard (2001). Preferentially a medium intended for use in susceptibility testing should be utilized. Recently a workshop funded by the EU proposes a set of guidelines for susceptibility testing of bacterial pathogens of importance in aquaculture (Alderman and Smith 2001). The proposed method for testing of *F. psychrophilum* is essentially the same as developed and used in Paper 1.

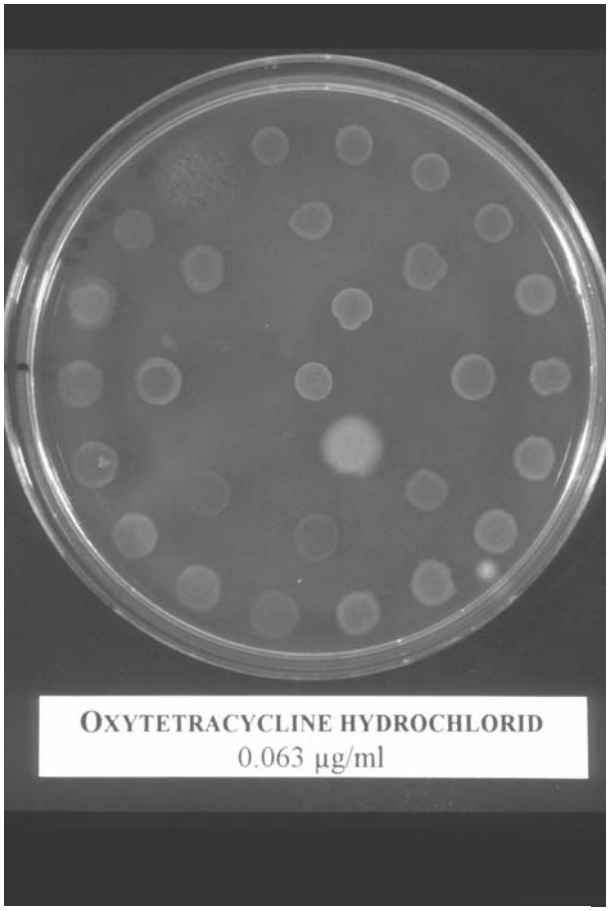


Fig. 8
MDMH agar plate containing 0.063 µg OTC/ml inoculated with control strains in the centre (surrounded by orange *F. psychrophilum* isolates).

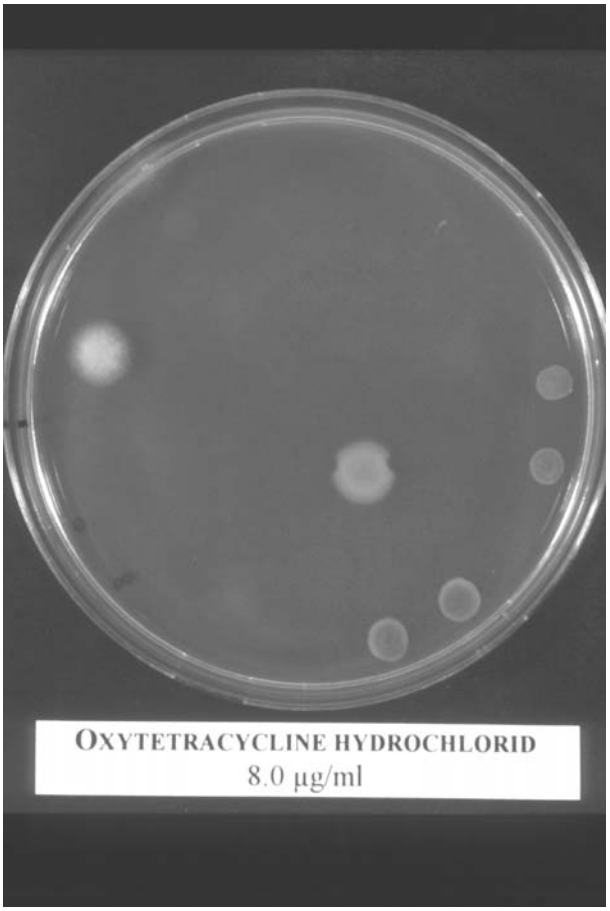


Fig. 9
MDMH agar plate containing 8.0 µg OTC/ml – only five of the tested *F. psychrophilum* isolates and the *Pseudomonas aeruginosa* control strain grow at this concentration.

2.2.3 Plasmids

Screening *F. psychrophilum* for plasmid content yielded a very consistent picture as the majority carried only one plasmid of approximately 3.3 kb (Fig. 10). No correlation to antimicrobial resistance could thus be established. These findings are consistent with other reports of plasmid profiles in *F. psychrophilum* (Lorenzen et al. 1997, Chakroun et al. 1998, Kroon and Wiklund 1998, Madsen and Dalsgaard 2000).

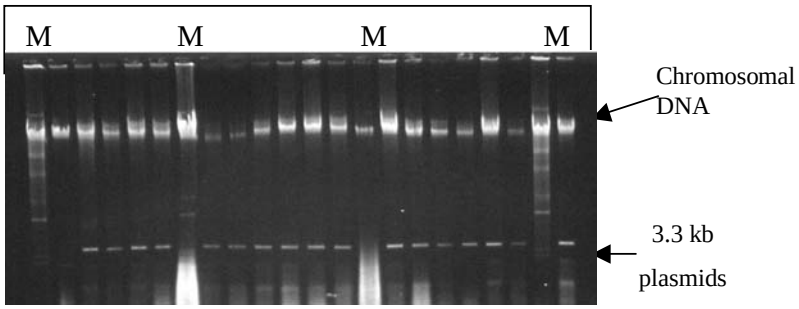


Fig. 10
Example of a *F. psychrophilum* plasmid screening.
M = marker (*E. coli* V 517)

2.2.4 Integrons

Using the int F and int B primers, most *F. psychrophilum* isolates apparently contained integrons (Fig. 11); some isolates even two or three integrons of different sizes (Fig. 12). This correlates with the finding that almost all *F. psychrophilum* isolates are resistant to S/T. Oddly enough, even the type strain that was less resistant to S/T was tested positive for an integron. Representative PCR product sizes were sequenced but only few Gene Bank sequences aligned well to the product when BLAST searches were done. In addition, no 59 base pair elements (Fig. 3) were seen and even the conserved segments of the integrons could not be recovered. Sequencing of equal and different sized PCR products were repeated but with the same result. Finally, PCR with a different primer set (qacEΔ1 F and sul1 B) specific for the 3' conserved region yielded no PCR product. As some of the sequences producing significant alignments were part of the 23S rRNA genes from *Mycoplasma*, *Ralstonia* and various Gram-positive bacterial species, it seemed reasonable to conclude that the original integron primers recognised a region on the *F. psychrophilum* genome independent of any integrons – probably the 23S region. Thus, none of the tested *F. psychrophilum* isolates contained type 1 integrons after all.

2.2.5 Tetracycline resistance determinants

Screening of all 63 oxytetracycline resistant *F. psychrophilum* isolates yielded only two positive reactions (both Tet C). Because of the phylogenetic relationship to Gram-positive bacteria screening of ten isolates to examine whether they contained Tet K, L, M, O and S (Table 1) was carried out by Yvonne Agersø at The Danish Veterinary Laboratory but without any positive

reactions (Paper 4). Thus, the genes coding for tetracycline resistance in *F. psychrophilum* are still largely unknown. No other reports of tetracycline determinants or other resistance genes in *F. psychrophilum* have been published so far.

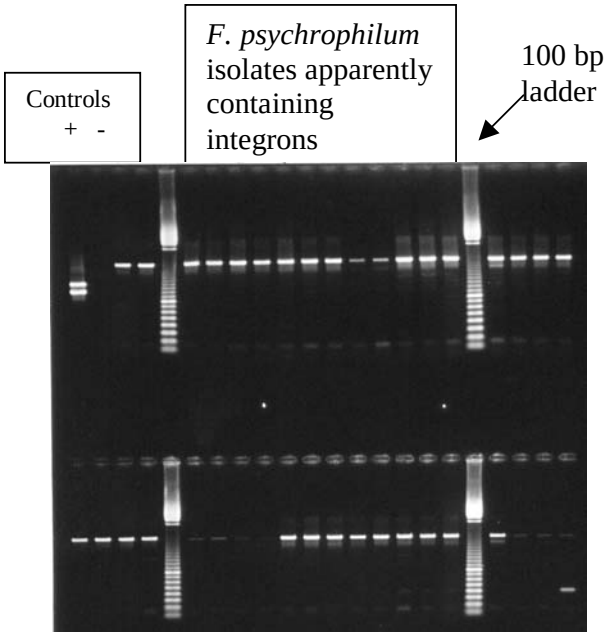


Fig. 11
Integron screening using int F and int B primers and a *Salmonella typhimurium* DT104 containing two integrons as a control.

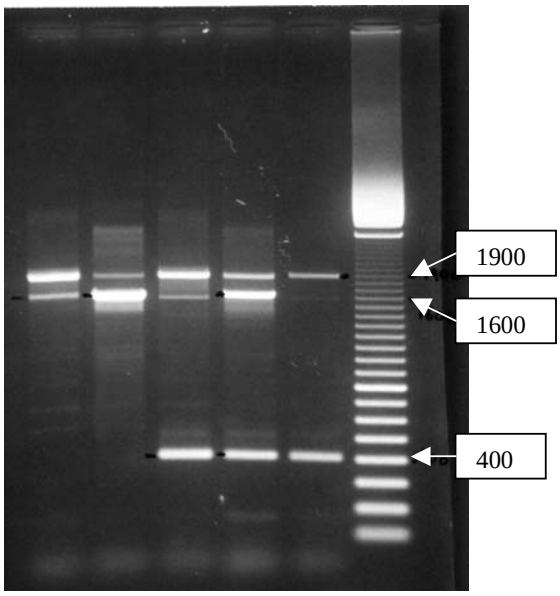


Fig. 12
F. psychrophilum isolates apparently containing integrons of varying sizes (400, 1600 and 1900 bp)

2.2.6 Transfer experiments

Attempts to transfer OTC resistance plasmids from several motile *Aeromonas* species to *F. psychrophilum* recipients were unsuccessful (Paper 4).

2.2.7 Treatment trial - correlation between treatment efficacy and resistance

Investigations of antimicrobial agents and antimicrobial resistance have traditionally been separated in examination of chemical properties, pharmacodynamic studies and laboratory experiments obtaining either inhibition zone diameters or genuine MIC values. More recently, studies of implicated resistance genes have been published for several bacterial species but no reports have so far addressed the nature of resistance in *F. psychrophilum*. In addition treatment of different fish diseases are being recommended on an empirical background (Branson 2001). Apparently no studies have addressed the direct treatment effect of fish diseases caused by bacteria with different in vitro susceptibilities. The classical correlations of in vitro susceptibility data and treatment efficacy in relation to obtainable serum concentration have been done in warm-

blooded animals and now it has been verified in rainbow trout infected with *F. psychrophilum* (Paper 5).

2.3 Motile *Aeromonas* spp.

The taxonomy of the genus *Aeromonas* has undergone several changes during the years that will not be discussed further here (Austin et al. 1996). Motile mesophilic *Aeromonas* spp. are ubiquitous bacteria in natural aquatic environments (Noterdaeme et al. 1991, Fukuyama et al. 1993, Sugita et al. 1995, Chaudhury et al. 1996, Noterdaeme et al. 1996, Hänninen et al. 1997, Miranda and Castillo 1998, Goñi-Urriza et al. 2000), where some species are known to cause diseases in fish and other poikilotherms (Austin and Adams 1996, Aoki 1999) and others are described as opportunistic human pathogens (Janda and Abbott 1996, Borrell et al. 1998, Ko et al. 2000). In Denmark, motile aeromonads rarely cause disease neither in humans nor in farmed animals.

The motile *Aeromonas* spp. were isolated from blood-, PADE- and TYES-agar plates containing AMX and subsequently identified to the genus level based on several biochemical tests (Joseph and Carnahan 1994). Isolates were considered to be *Aeromonas* spp. if they were Gram-negative, oxidase- and catalase-positive rods, which were facultative anaerobic and fermented carbohydrates, sometimes producing gas. Included isolates were also nitrate reducing, positive for arginine dihydrolase and lysine decarboxylase, negative for ornithin decarboxylase and resistant to O/129. The PADE agar allowed the growth of several motile *Aeromonas* species besides *A. hydrophila*, some of which can be extremely difficult to distinguish by conventional biochemical methods, in particular if they originate from environmental sources (Okpowasili 1991). The *Aeromonas* spp. are inherently resistant to β-lactam antibiotics like AMX although some authors report a small number of ampicillin-susceptible *Aeromonas* spp. (Chaudhury et al. 1996). The OTC-, OXA-, S/T- and FLO-resistance levels of the species in question did not vary significantly when the type strains were tested, and it was thus decided to include all motile *Aeromonas* isolates in the investigation as seen in several other studies (Fukuyama et al. 1993, Noterdaeme et al. 1996, Miranda and Castillo 1998).

Goñi-Urriza et al. (2000) examined the resistance patterns of 138 motile *Aeromonas* spp. isolates originating from two European rivers using an agar dilution method. They reported resistance frequencies of 59 % for nalidixic acid (MIC > 8 µg/ml), 14 % for tetracycline (MIC > 4 µg/ml), 7 % for the sulfamethoxazole/trimethoprim combination (MIC > 40 µg/ml), and 2 % were resistant to chloramphenicol (MIC > 8 µg/ml). A separation into three species/complexes was done (*A. caviae*, *A. sobria* and *A. hydrophila*) but it was stated that the three *Aeromonas* complexes shared a similar antibiotic susceptibility pattern.

Chaudhury et al. (1996) investigated resistance patterns in 108 clinical and environmental *Aeromonas* spp. Resistance was defined as strains growing at a concentration of 5 µg/ml regardless of the antimicrobial agent in question. All isolates were susceptible to tetracycline, aminoglycosides, cefotaxime and ciprofloxacin. Reported resistance frequencies ranged from 1.8 % for chloramphenicol, 9.3 % for TMP, and 14.8 % for nalidixic acid to 88.9 % for ampicillin. 23 % of the resistant isolates could transfer all or some of their resistance markers to *E. coli*, ten isolates carried plasmids that could be mobilized and further 45 isolates could be cured of one or more of the resistances (Chaudhury et al. 1996).

Kontny and Thielebeule (1988) reported 80 % OTC resistant *A. hydrophila* isolates from river water (MIC ≥ 20 µg/ml), 72 % were resistant to chloramphenicol (MIC ≥ 30 µg/ml) and 10 % resistant to TMP (MIC ≥ 20 µg/ml).

DePaola et al. (1988) reported OTC resistance between 58 and 83 % in *A. hydrophila* from catfish and a freshwater environment where OTC was used routinely.

The resistance frequencies reported in Paper 2 were 69 % for OTC (MIC > 8 µg/ml), 43 % for S/T (MIC > 512/102 µg/ml), 20 % for OXA (MIC > 2 µg/ml), and one isolate was resistant to FLO (MIC > 8 µg/ml). The resistance frequencies reported among motile *Aeromonas* spp. in other investigations have been derived using arbitrarily set breakpoints and as the breakpoints vary comparing the results is difficult. Nevertheless the resistance frequency reported in Paper 2 for OTC seems rather high, especially when taken into consideration that OTC is no longer used in Danish freshwater fish farms. Also a relatively high frequency of S/T resistance was found compared to previous reports.

The OTC resistance was examined in respect to the genetic background (tetracycline resistance determinants) and the potential of transfer. The high S/T resistance was examined and results showed that S/T resistance could be explained mainly by presence of integrons (Paper 3).

The motile *Aeromonas* spp. isolated during the present study showed a wide variety of plasmid profiles but no simple correlation between plasmid content and resistance patterns was found. No plasmids were detected in 46 % of the isolates. Transfer of OTC resistance was seen to E. coli from 43 % of the OTC resistant motile *Aeromonas* spp. harbouring large plasmids (> 30 kb) (Paper 3). Hedges et al. (1985) investigated transfer of resistance plasmids from *A. hydrophila* to *E. coli* and classified some of the transferable plasmids in incompatibility groups. Noterdaeme et al. (1991) detected no plasmids in 40 % of *A. hydrophila* isolated from freshwater fish and fresh water. The remaining isolates carried various numbers of plasmids but no correlation to resistance patterns was detected. Rhodes et al. (2000) detected transfer of OTC resistance from only 6.6 % of OTC resistant mesophilic *Aeromonas* spp. isolated from fish farm hatchery tanks to an *E. coli* recipient. A tetracycline resistance transposons Tn*1721* was involved in the dissemination of the OTC resistance.

Screening of OTC resistant motile *Aeromonas* spp. for tetracycline resistance determinants showed that less than 30 % could be assigned to the determinants A (8.8 %), D (2.8 %) or E (18.1 %), consequently 70.3 % remained non-classified (Paper 3). DePaola et al. (1988) classified the tetracycline resistance determinants in the majority of *A. hydrophila* isolates from channel catfish. The resistance was assigned to Tet A (25 %) and Tet E (68 %). In addition Tet B was detected in three of 214 isolates (DePaola et al. 1993). Rhodes et al. (2000) detected only Tet A and unknown determinants other than B-E and G in OTC resistant mesophilic *Aeromonas* spp. isolated from fish farm hatchery tanks.

Integrons were found in 45 % of the motile *Aeromonas* spp. and all S/T resistant isolates harboured integrons (Paper 3). To my knowledge, no other reports on integrons in motile *Aeromonas* spp. has been published.

2.4 *Yersinia ruckeri*

Y. ruckeri causes enteric redmouth disease (ERM) primarily in salmonids but has been isolated from a wide range of fish species as well as environmental samples (Horne and Barnes 1999). In this study presumptive *Y. ruckeri* colonies were isolated from blood- or ROD-agar (Furones et al. 1993) and subsequently identified with a species-specific PCR method (Argenton et al. 1996), serology and a range of biochemical tests (Furones et al. 1993). Isolates were considered to be *Y. ruckeri* if they were fermentative Gram-negative, oxidase negative and catalase positive rods, which were positive for lysine and ornithine decarboxylase, negative for arginine dihydrolase, utilizes citrate (Simmons) and not producing indole. Both a slide agglutination test and a species specific PCR had to test positive.

Despite the large number of environmental samples processed, *Y. ruckeri* was only isolated from fish - both diseased and apparently healthy. None of the isolated *Y. ruckeri* carried resistance towards the tested antimicrobial agents (Paper 2). Resistance and R-plasmids have previously been described in *Y. ruckeri* (de Grandis and Stevenson 1985, Ledo et al. 1987, Rodgers 2001). It was possible to transfer plasmid mediated oxytetracycline resistance to one *Y. ruckeri* isolate from two motile *Aeromonas* isolates in vitro (Paper 4). *Y. ruckeri* as recipient of resistance plasmids was also reported by Klein et al. (1996). The reason that the present *Y. ruckeri* isolates were generally susceptible towards the antimicrobial agents used to control ERM and other bacterial diseases in Danish freshwater aquaculture is unknown, but demonstrates that bacterial species react differently to apparently similar exposures. The resistance patterns detected in *Y. ruckeri*, *F. psychrophilum* and the motile *Aeromonas* isolates from the same samples were different. This contrasts the findings of Kelch and Lee (1978) who reported very similar resistance patterns in various bacterial groups from freshwater environments.

2.5 *Aeromonas salmonicida*

Furunculosis is a disease of salmonids and a wide range of other fish species caused by *A. salmonicida* (Hiney and Olivier 1999). No clinical outbreaks of furunculosis were verified at the fish farms during the sampling period and no *A. salmonicida* isolates were obtained from fish or the environmental samples (Paper 1). Antimicrobial resistance is well known in *A. salmonicida* (Hedges et al. 1985, Inglis and Richards 1991, Adams et al. 1998). The antimicrobial susceptibility of previously collected Danish and foreign isolates was determined and the importance of plasmid-borne class 1 integron-encoded resistance genes on horizontal spread of antimicrobial resistance in *A. salmonicida* was emphasized (Schmidt et al. 2001).

Chapter 3

Antimicrobial agents and resistance

3.1 Antimicrobial agents

Antimicrobial agents are substances that at low concentrations kill or inhibit microorganisms. The term comprises both antibiotics that are produced by microorganisms and the chemotherapeutics, which are synthesized compounds. Antibiotics are produced commonly by soil microorganisms and probably represent a means by which organisms in a complex environment, such as soil, control the growth of competing microorganisms. The microorganisms that produce antibiotics useful in preventing or treating disease include bacteria (*Bacillus* and *Streptomyces*) and fungi (*Penicillium, Cephalosporium,* and *Micromonospora*) (Russel and Chopra 1996). Since the discovery of penicillin in the 1920s many antimicrobial agents have been developed and antimicrobial chemotherapy has played a vital role in the treatment of human and animal infectious diseases.

Antimicrobial agents can be divided into major groups according to their basic chemical structure (Table 2). These groups are also different in respect to mode of action, antimicrobial spectrum and resistance mechanisms. The antimicrobial agents included in this study (Table 2) represent most of the major groups but no aminoglycosides, macrolides or lincosamides were included, as these drugs have never been used in Danish aquaculture and also to limit the workload.

3.2 Resistance mechanisms

During the years of using antimicrobial agents resistant bacteria have been observed. Resistance is not a new event, in fact bacteria carried resistance even in the pre-antibiotic era (Smith 1967) but apparently the levels of resistance have increased during the past decades. At present, several infectious bacteria carry multiple resistances that render them difficult and expensive to treat. This is the case for some strains of *Staphylococcus aureus* - some resistant to all antibiotics - *Enterococcus faecalis, Neisseria gonorrhoeae, Haemophilus influenzae, Mycobacterium tuberculosis, Escherichia coli, Pseudomonas aeruginosa, Shigella dysenteria, Streptococcus pneumoniae* and *Acinetobacter*, all being important community or nosocomial pathogens (Levy 1998). The problems seen in human medicine with multiple resistant pathogenic bacteria have not been reported to the same extend in fish pathogenic bacteria. Indeed resistant bacteria exist in aquaculture but so far only few descriptions of pathogens resilient to therapy with all the commonly used antimicrobial agents have been published.

Two broad categories of antibiotic resistance are recognized: intrinsic and acquired. Intrinsic resistance is an inherent function, usually expressed by chromosomal genes that protects the bacterium from antibiotic action. An example could be beta-lactam resistance in *Aeromonas* spp. When resistance emerges in a previously susceptible bacterial population it is named acquired resistance and is the result of mutations in chromosomal genes or uptake of resistance genes often as plasmids or transposons (Russel and Chopra 1996), as seen in Fig. 13.

Escaping the effects of antimicrobial agents can also be achieved by other means than developing resistance in the traditional sense. Bacteria are often found on surfaces where they can

Table 2. Antimicrobial agents

Major class	Mode of action	Spectrum	Resistance	Representative
Aminoglycosides	Inhibit translocation or initiation of mRNA by binding to 30S ribosomal subunits and subsequently protein synthesis is blocked.	Narrow	Modification of the aminoglycocides by plasmid- or transposon-encoded enzymes. Altered target or impaired uptake due to chromosomal mutations.	Not included
Beta-lactams	Inhibit the final stage of peptidoglycan synthesis and cause membrane damage.	Intermediate	Outer membrane changes, beta-lactamase production or modification of penicillin-binding-proteins. Mediated by chromosomal mutations, plasmids or transposons.	**Amoxicillin** (AMX)
Diaminopyrimidines	Inhibit bacterial dihydrofolate reductase and consequently the nucleic acid synthesis.	Narrow	Both chromosomal-, plasmid- and transposon-mediated.	**Trimethoprim** in combination with Sulfadiazin (S/T)
Lincosamides	Inhibit peptidyl transferase activity of 50S ribosomal subunits.	Narrow	Plasmid-mediated inactivation.	Not included
Macrolides	Stimulate dissociation of peptidyl-tRNA from ribosomes by binding to 50S ribosomal subunits.	Narrow	Chromosomal mutations or plasmid mediated.	Not included
Phenicols	Inhibit peptidyl transferase activity of 50S ribosomal subunits.	Broad	Inactivation by plasmid- or transposon-encoded acetyltransferases.	**Florfenicol** (FLO)
Quinolones	Inhibit DNA gyrase and thus the bacterial DNA replication.	Narrow for first generation Broad for flour-quinolones	Point mutations in *gyr*A.	**Oxolinic acid** a first generation quinolone (OXA)
Sulfonamides	Structural PABA-analogues that interrupt nucleotide metabolism and thereby inhibit the nucleic acid synthesis.	Narrow	Both chromosomal- and plasmid-mediated.	**Sulfadiazin** in combination with Trimethoprim (S/T)
Tetracyclines	Bind to 30S ribosomal subunits and inhibit binding of aminoacyl-tRNA's to ribosomal acceptor sites.	Broad	Determinants encoding efflux proteins or proteins that modify the binding site. Chromosomal-, plasmid- or transposon-mediated (details in Table 1). Non-specific membrane changes.	**Oxytetracycline** (OTC)

Compiled from Russel and Chopra (1996)

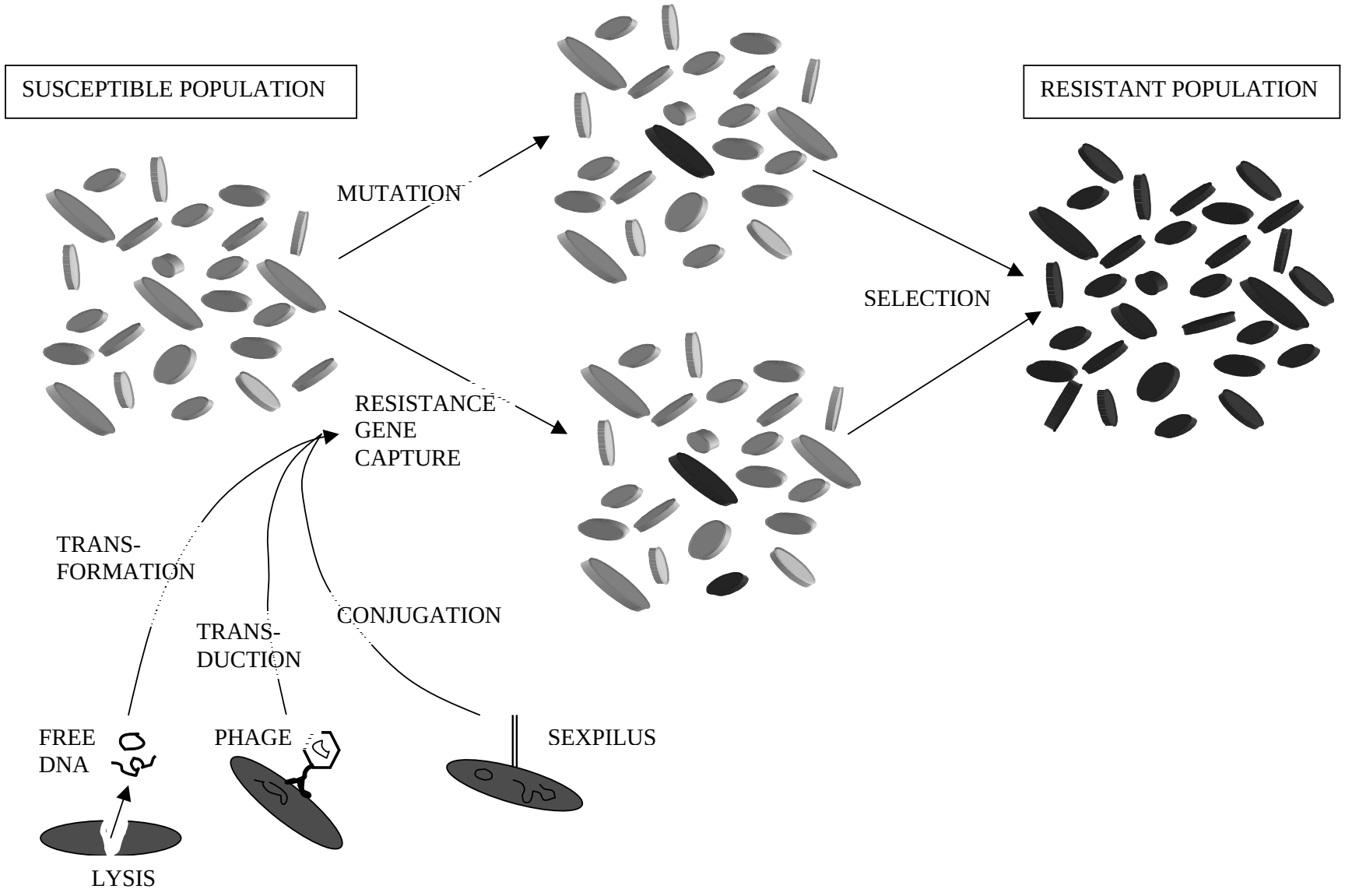


Fig. 13
Resistance development in a bacterial population.

form biofilms. These bacteria are embedded in an exopolysaccharide matrix that protects them against many antimicrobial agents on a population level (Lewis 2001). Some bacteria might reside in niches e.g. brain tissue where therapeutic concentrations of the antimicrobial agent never are reached. Finally, some bacteria are protected against a wide variety of antimicrobial agents due to the presence of multidrug transporters (Barnes et al. 1992, Putman et al. 2000). Resistance can be induced by other factors than antimicrobial agents. Accumulation of fish feed has been implicated in emerging OTC resistance (Kapetanaki et al. 1995) and thus questioning the contribution of antimicrobial residues and other factors on development of the detected resistance mentioned in previous studies. It also calls for development of alternative methods to predict the concentration of antimicrobial agents that could exert selection pressure as described by O'Reilly and Smith (1999) who used spectrophotometry and indirect conductance technology to determine a minimum concentration of OTC that produced a detectable effect on the growth or metabolism of an oxytetracycline sensitive strain (minimum effect level, MEC). They argue that the MEC can be used as a proxy measure of the minimum selection concentration and thus represent the first step in the development of environmental risk assessment models.

3.3 The aquatic environment

3.3.1 Fate of antimicrobial agents

Presence of antimicrobial agents in the aquatic environment can be a result of naturally produced antibiotics or may be introduced in the form of xenobiotics following manmade production and use of the agents. In general, pharmaceutical chemicals can enter the aquatic environment via two main routes (Halling-Sørensen et al. 1998):

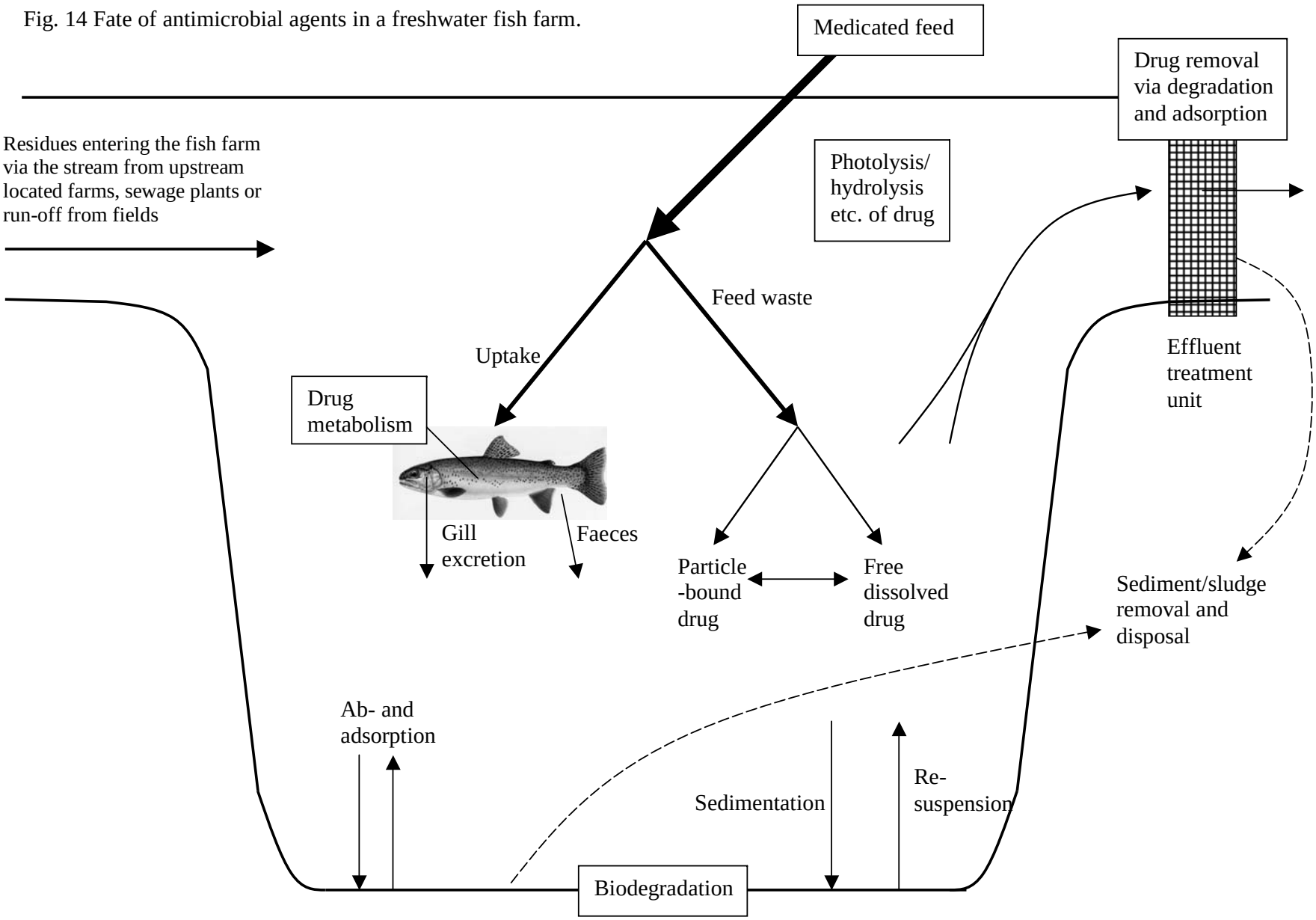
- The industrial route: Although the pharmaceutical industry works to stringent guidelines, some discharge in the sewage is inevitable. This wastage could enter the aquatic environment through a sewage treatment plant or indirectly via sewage sludge dispersed on fields and subsequent run off.
- The medical route: Pharmaceuticals used in hospitals, domestically or in veterinary medicine will eventually in some form and concentration end up in the environment. This goes for unaltered drugs excreted from treated individuals and excess drugs released to the environment in some form. A case of special interest or concern is treatment of animals in aquaculture where the drugs are administered more or less directly into the water via the feed and uneaten medicated feeds enter the sediment and water environment.

After introduction into the aquatic environment there are several possible fates of a xenobiotic (Richardson and Bowron 1985):

1. Total biodegradation to, e.g. carbon dioxide and water.
2. Partial degradation or metabolism - often to inactive metabolites.
3. Persistence - followed by wash-out/dilution or bioaccumulation.

The fate of antimicrobial agents in relation to a freshwater fish farm will depend on several factors. First of all, it will depend on the drug in question as physicochemical and pharmacodynamic properties vary. Factors such as solubility, pKa, bioavailability and conversion rate will influence the amount of drug released to the environment and the degradation to inactive metabolites. A schematic presentation of the pathways an antimicrobial agent can take in a fish farm is shown in Fig. 14.

Fig. 14 Fate of antimicrobial agents in a freshwater fish farm.



Eventually, non-degraded drug residues in the aquatic environment can exert their action on the environmental flora and fauna. In relation to antimicrobial agents the effect on the indigenous bacteria is most important as antimicrobial agents are designed to exert their action specifically or predominantly on bacteria. This means that therapeutic concentrations will have very limited effect on higher organisms and plants, whereas low concentrations (ng or µg/l) of antimicrobial agents are not expected to have bacteriostatic or -cid effect but might have the potential of selecting for resistance.

Few attempts have been reported to measure the concentration of antimicrobial agents in relation to freshwater aquaculture. Holten-Lützhøft (2000) detected OXA residues in the sediment from inlet, pond and outlet in a freshwater fish farm ranging from 0.4 to 0.8 µg/g at the inlet to 3.4 µg/g in the pond the day after medication and 1.6 µg/g 300 meters downstream 21 days after medication, but there was no clear correlation between sampling time/location and sediment concentration. Smith et al. (1994) used HPLC with a detection limit of 0.02 µg/ml to quantify OTC levels at a salmon hatchery. The fish farm effluent was passed through a circulating drum filter and effluent was sampled before and after the filter in addition to filter material. No OTC was detected in the effluent after the filter but in two samples OTC concentrations of 0.03 and 0.06 µg/ml were found in the water before filtering. In the filter material OTC was detected in all samples ranging from 0.57 to 13.0 µg/ml.
Hirsch et al. (1999) did not detect tetracyclines in samples from sewage treatment plant effluents, river surface water or ground water using preconcentration and HPLC with a detection limit of 0.05 µg/l of oxytetracycline, tetracycline, chlortetracycline and doxycycline. In contrast Watts et al. (1983) reported tetracycline concentrations in samples of river water around 1 µg/l using HPLC (cit. Richardson and Bowron 1985).

It should be noted that HPLC quantification of antimicrobial agents in the environment only addresses the amount of the drugs and not the biological activity as this is well known to decrease significantly due to various factors. Vaughan and Smith (1996) found that the biological activity of oxytetracycline hydrochloride was very significantly reduced in the presence of freshwater sediment. Although not addressed in this thesis, it is well known that complex formation of oxytetracycline with magnesium and calcium in seawater reduces the antibacterial effect of OTC. The uptake in fish intestines is decreased as well and this may lead to more OTC released to the environment that will end in the sediment. Whether this bound fraction of OTC will remain inactive or be released into the environment again is unclear. (Lunestad and Goksøyr 1990)

Regardless of the possible effects of releasing antimicrobial agents into the aquatic environment there is an wish on every organizational level to minimize the release of such xenobiotics. Several measures may reduce the occurrence of disease and subsequently the use of antimicrobial agents. Indeed a well-conducted vaccination programme on a national level has been seen to reduce antimicrobial drug use in Norway (Markestad and Grave 1997).

3.3.2 Antimicrobial resistance in the aquatic environment

Apparently resistant bacteria can be found in most aquatic environments. Various levels of resistant bacteria have been reported from polluted as well as unpolluted freshwater environments. Higher frequency of resistant bacteria from polluted than from unpolluted samples has been reported (Baya et al. 1986; Guardabassi et al. 1998) but the opposite has also been detected (Jones 1986, Miranda and Castillo 1998). As methods used and selected breakpoints differ between investigations it is not reasonable to compare results from different papers.

Miranda and Castillo (1998) determined resistance to various antimicrobial agents of motile *Aeromonas* spp. from Chilean fresh water by agar diffusion test using sensidisks. The highest number of tetracycline resistant *Aeromonas* spp. were detected from water classified as slightly polluted (14.3%), 10% tetracycline resistant bacteria were found in moderately polluted water and 8.9% in highly polluted water. This pattern was also seen for erythromycin whereas lower levels of resistance to the other tested antimicrobial agents were detected in the moderately polluted waters compared to the low- and high-polluted waters (Miranda and Castillo 1998).

Jones (1986) determined resistance levels in bacteria isolated from English rivers. The highest incidence of multiple resistance and resistance to individual antimicrobial agents were found in a remote upland tarn supposed to act as control site. The resistance levels were lower in a lake receiving effluent from a wastewater treatment plant and run off from fields, and the lowest incidence of resistance was detected directly in the effluent from the wastewater treatment plant.

Aquatic *Acinetobacter* spp. were resistance-typed by Guardabassi et al. (1999) detecting 50 % resistant to chloramphenicol, 27 % resistant to AMX, 26 % OTC resistant isolates, 26 % resistant to sulfamethoxazole and 7 % gentamycin resistant isolates. Guardabassi et al. (2000a) also demonstrated an increase in *Acinetobacter* spp. resistant to OXA from a stream receiving effluent from a fish farm following OXA medication at the farm.

McPhearson et al. (1991) reported similar effect on OTC resistance in bacteria isolated from catfish and aquaculture ponds where OTC treatment was undertaken.

This was emphasized by DePaola et al. (1995) who investigated bacteria from water and catfish and detected increased resistance levels during and after treatment with oxytetracycline. Levels increased from below 20% to around 40%, and the premedication level of resistance was reached again 21 days after end of treatment.

Spanggaard et al. (1993) detected resistance prevalences of 6 % to OTC (8 µg/ml) and 16 % to OXA (1 µg/ml) in bacteria isolated from an unpolluted stream. Among isolates from fish farms they reported 15 % resistance to OTC and 27 % for OXA, but no statistically significant differences were found.

Goñi-Urriza et al. (2000) detected 24.3% tetracycline resistant *Enterobacteriaceae* and 27.5% tetracycline resistant *Aeromonas spp.* in water samples from a river in Spain.

Paper 2 reports 69% of 313 aquatic isolates of motile *Aeromonas* spp. from Denmark to be OTC resistant. Average prevalence within the sampled culturable microflora was 4.8% OTC resistance, using the breakpoint level of 10 µg OTC/ml.

Schlotfeldt et al. (1992) reported a seasonal effect on the distribution of resistant and susceptible bacteria, but also noted a general decline in the number of resistant bacteria detected in the field practice of the Fish Health Service of Lower Saxony from 1986 to 1990. This decline might be caused by a restrictive drug prescription policy.

It is a matter of debate whether antimicrobial resistance will be reversed when the antimicrobial agent in question is discontinued. It has been argued that carrying a resistance plasmid requires energy and consequently the bacterium will loose the plasmid in the absence of a direct selection pressure in order to preserve energy. In contrast, Griffiths et al. (1990) showed that bacteria continue to carry the resistance plasmid but loose antibiotic resistance during long-term starvation. Although the bacteria retained the resistance genes, the enzymes required to detoxify the antimicrobial agents were either absent or inactive during starvation. Resistance returned when the bacteria were resuscitated (Griffiths et al. 1990). Chiew et al. (1998) reported that occurrence of streptomycin resistance in clinical Enterobacteriaceae remained high although the agent was used in diminutive amounts at the hospital in question. This could be associated with

cross-resistance to integron-encoded spectinomycin resistance. Other antimicrobial agents or heavy metals could also have the potential to exert an indirect selection pressure due to cross-resistance to a variety of agents. It has also been argued that even if most resistance-determining mutations and accessory elements (plasmids, transposons) bring about some fitness costs to the bacterium, those costs are minimised or disappear during subsequent evolution (Andersson and Levin 1999, Björkman and Andersson 2000). The results presented in Papers 1 and 2 regarding OTC resistance support that resistance is maintained. OTC resistance levels in *F. psychrophilum* were equally high in 1997 and 1998 when OTC use had been discontinued compared to the resistance levels in 1994 and 1995 (Paper 1). The OTC resistance level detected in motile *Aeromonas* spp. was rather high compared to other reports (Paper 2 and section 2.3).

3.4 Spread of antimicrobial resistance

Since gene recombination between *E. coli* strains was described in 1946 (Lederberg and Tatum 1946) the transfer, uptake and integration of DNA have been investigated in details and it is now known that gene transfer between bacteria can occur by three different mechanisms (Fig. 13):

Transformation is the process of uptake of a naked piece of double stranded DNA by a competent bacterial cell. The incoming DNA can be integrated into the bacterial chromosome and subsequently replicated with the recipient cell.

Transduction happens when the donor DNA is packed into the capsid of a transducing bacteriophage and injected into the recipient cell during a subsequent infection.

Conjugation is mediated by plasmids encoding their own transfer from donor to recipient. Transfer of the DNA occurs through sex pili in Gram-negative bacteria and by direct contact in Gram-positive bacteria. Conjugative plasmids carry the transfer (*tra*) operon that codes for all the functions needed for conjugation. Conjugative plasmids may also mediate the transfer of non-conjugative ones either by cointegration, retrotransfer or via mobilisation that requires a *mob*-gene in the non-conjugative plasmid (Trevors et al. 1987).

Spread of resistance genes can also be facilitated by other genetic structures as transposons (Mahillon 1998) and integrons (Hall and Collis 1995).

Transposons are transposable genetic elements that move from place to place in the chromosome and plasmids of a single bacterium. The simplest transposons are insertion sequences (Mahillon et al. 1999) that contain only the genetic information required for their own transposition. Larger ones (composite transposons) may include various genes. They can be conjugative and thus transfer horizontally between bacteria. They often mediate antimicrobial resistance.

Integrons are not capable of transposition between the chromosome and plasmids but are able to capture and integrate gene cassettes often consisting of one or more resistance genes flanked by insertion sequence (IS) elements. An integron can actively integrate newly acquired foreign gene cassettes into the bacterial chromosomal or plasmid DNA. Over 40 different antibiotic resistance genes have been found in integrons (Hall and Collis 1995, Rowe-Magnus et al. 2001).

The contribution of each mechanism to the gene exchange and spread in an aquatic environment is still mostly a matter of belief. Several reviews deal with this subject (Trevors et al. 1987, Coughter and Stewart 1989, Young 1993, Goodman et al. 1994, Hermansson and Linberg 1994, Lorenz and Wackernagel 1994, Ashelford et al. 1997, Dröge et al. 1999).

Transformation is probably not very common as DNA has a hard time persisting in the natural environments due to degradation by exonucleases. However, DNA can be quite stable in circular

forms or associated with soil components - and competent cells can take up this DNA (Miller 1998). In addition many bacteria isolated from the aquatic environment are naturally competent (Paul and Williams 1995). This leads to the belief that transformation might be more important as a means of gene exchange in natural environments than previously believed (Lorenz and Wackernagel 1994).

Transduction as a mechanism of gene transfer in the environment has the advantage over transformation that the DNA is protected inside the bacteriophage. In addition, concentrations of bacteriophages in fresh and marine waters can be up to 10^{11} phages/ml (Miller 1998), and in samples from the Adriatic Sea the visible infection frequency of the entire bacterial community was as high as 30% (Day and Marchesi 1996). Jiang and Paul (1998) detected transduction frequencies in the marine environment from $1.33x10^{-7}$ to $5.13x10^{-9}$, and suggested that transduction could be an important mechanism for horizontal gene transfer in the marine environment.

The role of conjugation in the spread of genes in the environment is debatable. Intracellular barriers, such as incompatibility between two plasmids residing in the same cell and restriction-modification systems that enable the recognition and degradation of incoming donor DNA, act to prevent transfer (Trevors et al. 1987). In contrast to this, Chandrasekaran et al. (1998) claim that horizontal transfer of plasmid-encoded genes is the primary reason for the dissemination of resistance genes in the environment. This statement is not founded on direct scientific evidence as comparable quantification of the involved mechanisms in a natural environment has yet to be done, but it is a fact that most papers on resistance gene transfer deals with conjugation, and it was suggested already in 1975 that resistance plasmids (R factors) were widely distributed in natural environments (Aoki 1975) and a large fraction of aquatic bacteria had the capacity to act as recipients of plasmid DNA (Genthner et al. 1988).

It has been shown that plasmid transfer between bacteria occurs in a variety of natural environments, e.g. river water (Trevors and Oddie 1986, Bale et al. 1988, Paper 4), lake water (O'Morchoe et al. 1988), sewage effluent (Altherr and Kasweck 1982), seawater (Goodman et al. 1993, Goodman et al. 1994, Kruse and Sørum 1994, Dahlberg et al. 1998), marine sediments (Paul et al. 1991, Sandaa et al. 1992) and soil (Lafuente et al. 1996, Pukall et al. 1996).

In addition, some environmental hot spots for gene transfer have been described in the literature. Higher transfer efficacy was seen in the residuesphere (Dröge et al. 1998, Sengeløv et al. 2000), in biofilm (Angles et al. 1993, Hausner and Wuertz 1999) and the gut of microarthropods (Hoffmann et al. 1998) and probably this is valid in the gut of other animals e.g. fish.

Concluding remarks

The resistance pattern of the fish pathogenic bacterium *F. psychrophilum* was assessed in vitro using a standardised method based on existing guidelines but modified according to Paper 1. The occurrence of tetracycline resistance in 387 Danish isolates was determined as well as their resistance towards other antimicrobial agents used in Danish freshwater aquaculture. Resistance was widespread towards OTC (68%), OXA (66%) and S/T (98%). AMX resistance was absent among the early isolates but occurred widespread (approx. 40%) in 1997 and 1998. No resistance to florfenicol was detected.

Resistance of total culturable bacteria in a freshwater environment to antimicrobial agents used to treat bacterial fish diseases in Danish aquaculture was investigated by differential plating. The only detected significant effect was a higher number of OTC resistant bacteria at the outlet of fish farms compared to the inlet (Paper 2). In hindsight the author find this method usable but not very useful for routine monitoring of the resistance. Instead I incline to use the other approach of isolating specific bacterial species from relevant locations and comparing their resistance patterns (Papers 2 and 3). It should be noted that bacterial species have varying ability to develop resistances and thus more than one species should be used concurrently.

Resistance patterns of both motile *Aeromonas* spp. and *F. psychrophilum* isolated from the aquatic environment support the results from the differential plating in that more OTC resistant bacteria are present at the outlet. In addition, a similar pattern was detected for the motile *Aeromonas* spp. regarding resistance to sulfadiazine/trimethoprim and oxolinic acid. The increased resistance was apparently localised to the single fish farm environment, as no accumulation of resistant bacteria was observed further down the stream by either of the methods.

To characterise the nature of the resistance (Paper 3 + 4) all isolates were examined for their plasmid content without any obvious correlation to the resistance detected. Bacteria resistant to oxytetracycline were screened for specific tetracycline determinants (A-E). In contrast to earlier reports (comprising mainly clinical isolates) only 30 % of the oxytetracycline resistance found in motile *Aeromonas* spp. were assigned to one of the tested determinant classes and only two of 63 oxytetracycline resistant *F. psychrophilum* were classified (Tet C). No resistant *Y. ruckeri* isolates were detected among the 134 identified isolates and no *A. salmonicida* was isolated during the sampling period. Bacteria resistant to sulfadiazine/trimethoprim were examined for class 1 integrons that always carries sulfonamid resistance and in addition have the ability to integrate other resistance genes as gene cassettes. No integrons were detected in *F. psychrophilum* but the occurrence of integrons could account for the detected resistance to sulfonamides in the motile *Aeromonas* spp. The involved gene cassettes were sequenced and shown to contain genes coding for resistance to trimethoprim, aminoglycosides and chloramphenicol.

Transfer of resistance in vitro has been assessed both by filter matings, in simple microcosms and in membrane filter chambers (Papers 3 and 4). By filter mating motile *Aeromonas* spp. with large plasmids yielded 17 successful conjugal transfers out of 40 to *E. coli*. Resistance to antimicrobial agents other than OTC (used for selection) was co-transferred and could be assigned to integrons containing gene cassettes (Paper 3). These gene cassettes coded for resistance to

combinations of aminoglycosides, trimethoprim or chloramphenicol in addition to the sulfonamid-resistance inherent to class 1 integrons (Paper 3). Transfer was also shown between motile *Aeromonas* spp. and to *Y. ruckeri* but no transfer was seen to *F. psychrophilum*. Transfer of R-plasmids was demonstrated in situ. Membrane filter chamber submersed into the stream from which the isolates originated were used to mimic the natural conditions, and transfers were shown from selected motile *Aeromonas* spp. and *A. salmonicida* to *E. coli* and between motile *Aeromonas* isolates.

The effect of treatment towards certain fish pathogenic bacteria in vitro was considered in Papers 1 and 2 and subsequently in vivo effect of RTFS treatment was examined in Paper 5 using an experimental infection model and feed medication with OTC. Successful treatment was shown in the fish infected with an in vitro suceptible *F. psychrophilum* isolate, whereas limited or no effect of the treatment was seen in fish infected with less susceptible *F. psychrophilum* isolates. This study stresses the importance of doing susceptibility testing to assess treatment efficacy.

Perspectives for future research

As resistance development or acquisition vary in different species although they reside in the same environment a set of three or four indicator bacteria to monitor resistance is necessary. Selection of such species and an international consensus regarding use of the same organisms could provide a strong tool in future risk assessments. Likely candidates to include from the freshwater environment could be motile *Aeromonas* spp., *Acinetobacter* spp., and selected *Enterobacteriaceae* and *Enterococcus* spp. – preferably a defined species from each of these or other groups.

Resistance monitoring in relation to seawater aquaculture will require different indicator bacteria. Investigating the conditions in seawater fish pathogenic and environmental *Vibrio* species could be proposed for further studies.

Quantification of antimicrobial residues in the ponds and at the outlet of freshwater fish farms in relation to medication is sparse. Further development of sufficiently sensitive methods for measuring the presumably low concentrations might be necessary. Knowing the concentration of the antimicrobial agents in the exposed environments could be useful for risk assessment if it is possible to establish a method to determine “no effect level” of antimicrobial agents in relation to resistance development. This could either relate to a set of selected bacterial species or to the effect on a complex bacterial community.

Although much work has been done regarding transfer of resistance genes in the natural aquatic environments, transfer in more life-like scenarios should be examined further. This could be done examining whole bacterial communities or using labelled bacteria or labelled resistance genes to monitor the events at single-cell level. The areas that could be addressed are transfer in a mixed bacterial community with natural bacterial numbers and interaction with the indigenous aquatic bacteria, plants and animals e.g. transfer experiments in the gut of fish.

The genetic background of the resistance observed in the *F. psychrophilum* isolates is unknown. Resistance plasmids in *F. psychrophilum* is still a possibility that should be looked at as well as transfer experiments with *F. psychrophilum* as putative donor – screening with *E. coli* or *P. putida* as donors could be attempted, but it will probably be necessary to use more closely related species, e.g. another *F. psychrophilum* isolate made resistant to rifampicin to facilitate counterselection.

There will clearly be a need to continue research in ways to avoid or limit antimicrobial resistance development in general and in relation to aquaculture production to assure future treatment efficacy. This could be achieved by development or improvement of ways to control bacterial diseases other than treatment with known antimicrobial agents. Examples include 1) preventive measures as effective vaccines, probiotics and/or immunostimulants supplied directly in the water or via the feed, 2) development of new classes of antimicrobial agents with unique antibacterial mechanisms, 3) simultaneous treatment with classic drugs in combination with specific inhibitors of the resistance in question, 4) alternative antibacterial treatments using e.g. bacteriophages, and 5) development of drugs that stop or reduce the spread of resistance genes by impeding conjugation or other of the known transfer mechanisms.

Acknowledgements

My sincere thanks to my supervisors professor Jens Laurits Larsen and senior scientist Inger Dalsgaard for inspiring discussions, sharing of your knowledge and critical review of articles and this thesis.

Thanks to:

- All the colleagues and technicians at the Department of Veterinary Microbiology, especially at the Laboratory of Fish Diseases, and at the Fish Disease Laboratory, Danish Institute for Fisheries Research. Thanks for your company, for always being helpful and making the laboratory a pleasant place to work.

- Anja S. Schmidt for the many hours we have spent together working in the lab, driving to Jutland, sampling at the fish farms in any kind of weather, discussing microbiology, listening to music and talking about life.

- Farah S. Bahrani for your good mood and excellent technical work.

- Lone Madsen for help and discussions and reviewing this thesis.

- Tom Wiklund for help, discussions and some rather exhausting squash matches.

- Andreas Petersen and Luca Guardabassi for nice company in our shared office.

- Karl Pedersen for help and supervision in the initial phases of the project.

- Owners and workers at the fish farms for interest in the project and for pleasant collaboration.

- Veterinarians Jes Nissen, Svend Kirkeby and Henrik Korsholm for sending in diseased fish and bacterial cultures.

- Frank Møller Aarestrup, Lars Bogø, Dorthe Sandvang and technicians at the Danish Veterinary Laboratory for help with primers and reference strains for PCR to detect tetracycline resistance determinants and integrons and for sequencing of the PCR products.

- Søren Sørensen and Lars Hestbjerg at Copenhagen University for suggestions regarding resistance transfer experiments and donation of recipient strains.

This research was supported by a scholarship from the Royal Veterinary and Agricultural University and funded in part by The Ministry of Food, Agriculture and Fisheries.

Til slut en meget stor tak til min dejlige familie, som altid har vist interesse for hvad jeg gik og lavede og som jeg virkelig nyder at være sammen med. Ikke mindst mine elskede børn og min enestående kone.

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The pre-print editions of paper three, four and five are included in this PDF. The papers, including paper 1 and 2, can be found here:

Paper 1: Bruun, M.S., Schmidt, A.S., Madsen, L., Dalsgaard, I. 2000. Antimicrobial resistance patterns in Danish isolates of Flavobacterium psychrophilum. Aquaculture. 187, 201-212. DOI: 10.1016/S0044-8486(00)00310-0

Paper 2: Schmidt, A.S., Bruun, M.S., Dalsgaard, I., Pedersen, K., Larsen, J.L. 2000. Occurrence of Antimicrobial Resistance in Fish-Pathogenic and Environmental Bacteria Associated with Four Danish Rainbow Trout Farms. Applied and Environmental Microbiology. 66, 4908-4915.

Paper 3: Schmidt, A.S., Bruun, M.S., Dalsgaard, I., Larsen, J.L. 2001. Incidence, Distribution, and Spread of Tetracycline Resistance Determinants and Integron-Associated Antibiotic Resistance Genes among Motile Aeromonads from a Fish Farming Environment. Applied and Environmental Microbiology. 67, 5675-5682. DOI: 10.1128/AEM.67.12.5675-5682.2001

Paper 4: Bruun, M.S., Schmidt, A.S., Dalsgaard, I., Larsen, J.L. 2003. Conjugal Transfer of Large Plasmids Conferring Oxytetracycline (OTC) Resistance: Transfer between Environmental Aeromonads, Fish-Pathogenic Bacteria, and Escherichia coli. Journal of Aquatic Animal Health. 15, 69-79. DOI: 10.1577/1548-8667(2003)015<0069:CTOLPC>2.0.CO;2

Paper 5: Bruun, M.S., Madsen, L., Dalsgaard, I. 2003. Efficiency of oxytetracycline treatment in rainbow trout experimentally infected with Flavobacterium psychrophilum strains having different in vitro antibiotic susceptibilities. Aquaculture. 215, 11-20. DOI: 10.1016/S0044-8486(01)00897-3

Incidence, distribution and spread of tetracycline resistance determinants and integron encoded antibiotic resistance genes among motile aeromonads from a fishfarming environment

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Running title: Tetracycline resistance determinants and integrons in aquatic aeromonads

ABSTRACT

A collection of 313 motile aeromonads isolated at Danish rainbow trout farms was analyzed to identify some of the genes involved in high levels of antimicrobial resistance found in a previous field trial (37), the predominant resistance phenotype (37%) being a combined oxytetracycline (OTC) and sulphadiazine/trimethoprim (S/T) resistance. Combined sulphonamide/trimethoprim resistance (135 isolates) appeared closely related to the presence of a class 1 integron (141 strains). Apart from 23 isolates with "empty" integrons without inserted gene cassettes, we found four different combinations of resistance gene cassettes, in all cases including a dihydrofolate reductase gene and a downstream aminoglycoside resistance insert (87 isolates), and occasionally an additional chloramphenicol resistance gene cassette (31 isolates).

As far as OTC resistance was concerned, only 66 (30%) out of 216 resistant aeromonads could be assigned to resistance determinant classes A (19), D (6) or E (39); three isolates contained two tetracycline resistance determinants (AD, AE, DE, respectively).

Forty OTC-resistant isolates containing large plasmids were selected as donors in a conjugation assay, twenty-seven of which also contained a class 1 integron. Out of 17 successful R-plasmid transfers to *E. coli*- recipients, the respective integrons were co-transferred along with the tetracycline resistance determinants in 15 matings. Transconjugants were predominantly *tetA*-positive (10/17), and contained class 1 integrons with two or more inserted antibiotic resistance genes. While there appeared to be a positive correlation between conjugative R-plasmids and *tetA* among the OTC-resistant aeromonads, *tetE* and the unclassified oxytetracycline resistance genes as well as class 1 integrons were equally distributed among isolates with and without plasmids. These findings indicate the implication of other mechanisms of gene transfer besides plasmid transfer in the dissemination of antibiotic resistance among environmental motile aeromonads.

INTRODUCTION

The motile aeromonads represent a group of ubiquitous aquatic microorganisms, which are not generally considered to be primary human pathogens (16). Some species, however, have been isolated from local or generalized human infections (8, 16, 21). By contrast, many members of the group are recognized as primary pathogens to a wide range of cold-blooded animals, in particular to fish (4, 6, 42). In temperate regions with mainly salmonid production, motile *Aeromonas* species are not commonly associated with disease outbreaks in aquaculture, and thus not directly targeted by treatment with antimicrobial agents.

However, freshwater fish farming does seem to have an impact on environmental *Aeromonas* spp., as indicated by a previous investigation of antimicrobial resistance at four Danish rainbow trout farms (37). Water-, sediment- and fish samples were examined, and because of their ubiquitous distribution in the freshwater environment, the motile aeromonads were selected as bacterial indicators. In addition, members of the genus *Aeromonas* readily develop single or multiple antimicrobial resistance phenotypes (11, 13, 19, 24-26), and R-plasmids are commonly found (1, 3, 5, 14, 20, 30). Thus, they were well-suited for monitoring the incidence of antibiotic resistance, as well as for investigating the conjugative spread of resistance genes in these settings.

Significantly higher proportions of antibiotic resistant motile aeromonads were detected in the effluent of fish farms compared to their inlets (37). In particular, many of the isolates were resistant to high levels of oxytetracycline (OTC), a combination of sulphadiazine/trimethoprim (S/T) or both (37%). Potentiated sulphonamides and oxolinic acid (OXA) are the only antimicrobials licenced for therapeutic use in Danish aquaculture, while the usage of oxytetracycline is restricted, requiring dispensation in every case. The small amounts of OTC used in the fish farms thus did not correlate with the observed high OTC-resistance frequencies among environmental *Aeromonas* isolates (37). By contrast, the administration of S/T in fish farms during bacterial disease outbreaks might have promoted the emergence of S/T-resistance in the vicinity of the farms.

Both types of resistance have been reported to be encoded by transferable plasmids within the genus *Aeromonas* (1, 3, 8, 15). Several classes of tetracycline resistance determinants have been described on R-plasmids within this group of bacteria (1, 2, 5, 9, 10, 32). Tetracycline resistance genes are frequently part of transposons, which are able to change their location within the cell, and thus achieve increased mobility e.g. by inserting into conjugative plasmids (44, 45). Consequently, we decided to investigate the occurrence and distribution of the tetracycline resistance determinants class A to E among the motile *Aeromonas* isolates, comparing OTC-resistant isolates with different plasmid profiles and origins.

Furthermore, the aeromonads were screened for the presence of class 1 integrons, in order to elucidate the genetic background of the high S/T-resistance level. The 3' conserved segment of the integron includes the *sul1* and *qacEΔ1* genes, encoding sulfonamide- and quarternary ammonium compound resistance, respectively. The 5' conserved segment contains the genes encoding integration and expression of varying numbers of gene cassettes, comprising the variable part of the structure. The gene cassettes usually are antimicrobial resistance genes, and dihydrofolate reductase (*dhfr*) genes conferring trimethoprim resistance are common (23, 31).

Only few studies have to date addressed the prevalence of class 1 integrons among environmental bacteria (29, 33), and to our knowledge, this is the first report of class 1 integrons within the widely distributed motile *Aeromonas* species.

Even though class 1 integrons are transposition-defective, they are often plasmid-borne as they are mobilizable in association with a functional transposon, or by transposition proteins supplied *in trans* (1, 9, 10, 34, 44, 45). Thus, their presence might indicate, whether horizontal gene

transfer has occurred among the aeromonads found in and around the sampled fish farms, and associated antibiotic resistance genes could explain some of the observed resistance patterns.
Subsequent conjugation assays were used to assess the transferability of both tetracycline resistance determinants and class 1 integrons on conjugative plasmids.

MATERIALS AND METHODS

Bacterial isolates and MIC-testing.

Between October 1997 and February 1999, we sampled four fish farms situated along a Danish stream on eleven occasions with monthly intervals. Farm 1 was located furthest upstream and thus received no effluents from other fish farms. However, the stream had previously received effluents from a sewage treatment plant and some agricultural areas. Farm 2 was situated close to the outlet of the adjacent upstream fish farm, and farm 4 was one of the last farms downstream. Water- and sediment samples were collected from inlet, outlet, and a pond of each farm as earlier described (37). Samples from gills and skin mucus of two to four fish per farm were also included. The processing and culturing of the samples is described in detail in a previous paper (37). Random colonies were selected and screened for presumptive *Aeromonas* isolates with a panel of tests comprising Gram reaction, motility, morphology, catalase and oxidase production, oxidative/ fermentative utilization of glucose and susceptibility to O/129 (17). In order to establish a phenospecies identification of the isolates, the following biochemical tests were performed: beta-haemolysis, arginine dihydrolase, lysine- and ornithine decarboxylase, esculin hydrolysis, gas production from glucose, Voges-Proskauer test, acid from sucrose/ lactose/ salicin/ arabinose/ cellobiose (modified after (17)).
All isolates and transconjugants were tested in a standardized agar dilution assay (27, 37) in order to determine their MIC-values towards several antimicrobial agents currently used in Danish aquaculture, including oxytetracycline (OTC), sulphadiazine/trimethoprim (S/T) and oxolinic acid (OXA). The following breakpoints were established (37): isolates were considered to be OTC-resistant when having MIC values >8 μg ml^{-1} (sensitive isolates 0.125 to 1.0 μg ml^{-1}), S/T-resistant when exhibiting MIC values >512/102 μg ml^{-1} (sensitive isolates: 0.5/0.1 to 8/1.6 μg ml^{-1}), and OXA-resistant with MIC values >2 μg ml^{-1} (sensitive isolates: 0.125 to 1.0 μg ml^{-1}).

Plasmidprofiling

All aeromonads and *E. coli* transconjugants were screened for their plasmid content, applying the alkaline lysis method described by Kado and Liu (18), followed by agarose gel electrophoresis. We used the 4.0 version of GelCompar (Applied Maths, Kuijper, Belgium) to analyze the resulting plasmid profiles.

Conjugational gene transfer

All OTC-resistant isolates with large plasmids were included as putative donors in a filtermating assay, in order to detect the transfer of R-plasmids to a rifampicin-resistant *Escherichia coli* strain, CSH26Rf (39). Overnight cultures of donor and recipient were adjusted to OD_{600}=0.5 with fresh veal infusion broth. Equal volumes (50μl) of each culture were mixed on a sterile 0.2μm nitrocellulose filter (Sartorius AG, Goettingen, Germany) placed on a veal infusion agar plate (Difco) and incubated at 20˚C overnight. Cells were washed off the filter by vortexing in 10 ml sterile 0.9% NaCl solution, and appropriate tenfold dilutions were prepared. From each dilution, 100μl aliquots were spread on selective agar plates, containing 20 μg ml^{-1} oxytetracycline, 100 μg ml^{-1} rifampicin (Bie&Berntsen, Rødovre, Denmark) or both. Donor and recipient were also plated

on the double selective plates for mutant detection. All assays were run in duplicate. The identity of transconjugants was confirmed biochemically (oxidase production, amino acids, Voges-Proskauer reaction), and they were screened for the presence of plasmids as described above.

Detection of tetracycline resistance determinants

We decided to test all OTC-resistant isolates for tetracycline resistance determinants A-E because they include the classes which are most commonly described in aeromonads (1, 2, 5, 9, 10) and frequently associated with R-plasmids (1, 9, 32). A multiplex PCR-assay was used according to Guardabassi et al. (13). *E.coli* strains containing the respective tetracycline resistance genes were included (classA: NCTC50078; classB: HB101/pRT11; classC: DO7/pBR322; classD: C600/pSL106; class E: HB101/pSL1504). Primers are listed in Table 1. All isolates and transconjugants with MIC-values exceeding 8 μg ml^{-1} OTC were tested (Table 2).

Analysis of antimicrobial resistance genes associated with integrons

All isolates and transconjugants were screened for the presence of class 1 integrons with specific primers targeting the conserved 5' and 3' segments of the structure as previously described (36). Thus, the size of a PCR product depends on the number and size of the inserted gene cassettes (Fig. 1+2). *Salmonella typhimurium* DT104 (9616368) was the positive control strain. PCR-products were purified (S-400 HR MicroSpin™ Columns, Amersham Pharmacia Biotech, Uppsala, Sweden) and sequenced. The nucleotide sequence was determined in both senses of the DNA, using a cycle sequencer 373A (Applied Biosystems, Perkin Elmer, Foster City, CA, USA) as reported earlier (36). With five different sizes ranging between about 150bp (no insert) and 2900bp (Fig. 1), two to three representatives of each amplicon were sequenced. Subsequently, a suitable DNA restriction enzyme (MboII) was employed to determine, whether equally sized integron amplicons contained the same gene cassettes. Restriction cutting and purification of DNA was performed as described (35), and the resulting fragments were separated by agarose gel electrophoresis.

The 1550bp amplicons were most prevalent and invariably appeared to contain a *dhfrI*- and a downstream *ant(3")1a* insert. As these resistance gene cassettes were found alone or in combination in every amplicon type (Fig.2), a specific PCR-assay targeting the *dhfr1* and *ant(3")1a* genes was used to detect their presence and order within all PCR-products (38)(Table 1).

A separate primerset was used to investigate the 3' conserved segment of the class 1 integrons (Table 1), containing the *qacE*Δ and *sul1* genes (36), in order to detect defective copies with an incomplete *sul1* gene, which were frequent findings in a study of aquatic bacteria by Rosser and Young (33).

Statistical methods.

The proportions of antibiotic resistance and the respective detected genes among the *Aeromonas* isolates were computed for each fish farm and all sampling sites. A logistic regression model was employed (proc genmod in SAS version 6.12, SAS Institute Inc., Cary, NC, USA) to detect differences between resistance rates from in- and outlet as well as differences between fish farms. In a few cases, where the logistic regression model did not describe the data well, χ^2tests (with continuity correction) were performed instead.

RESULTS

Resistance phenotypes

Three-hundred thirteen isolates belonged to one of the motile, mesophilic species within the genus *Aeromonas*. The most prevalent phenospecies identified were *A. hydrophila* (35.3%), *A. bestiarum* (19%) and *A. veronii* biovar *sobria* (15.3%). Fifteen percent of the isolates could not be reliably assigned to one phenospecies. Antibiotic resistance patterns did not vary significantly between the identified phenospecies (data not shown).

A total of 216 *Aeromonas* isolates (69%) were resistant to OTC, while 135 (43%) displayed S/T-resistance (37). Sixty three isolates (20%) were OXA-resistant. Multiresistance was common, as 151 isolates (48%) carried at least two additional antibiotic resistance traits besides AMX-resistance, which is implicit in the following.

The predominant multiresistance phenotype was OTC-S/T-resistance (89 isolates, 28%), followed by OTC-OXA (31 isolates, 10%) and OTC-OXA-S/T (28 isolates, 9%). Most resistance patterns were equally likely to occur in all farms. One exception was OTC-S/T, farm 3 isolates being less likely to have this phenotype (11%) than isolates from farm 1 (38%), 2 (33%) and 4 (25%). However, this difference was non-significant. Twenty-four percent of the aeromonads (75 isolates) were sensitive to all of the four remaining antibiotics. The results from the statistical analysis of the overall resistance data were earlier described in detail (37), and only a few significant effects were included here (Table 2).

Plasmidprofiles and conjugative transfer of resistance genes

The plasmid content of the 313 aeromonads did not seem to vary between the different phenospecies (data not shown). Onehundred forty-four (46%) isolates did not contain any plasmids, while 16% did harbour at least one large (>30kb) plasmid (profile A) (Table 3). The GelCompar analysis yielded three additional clusters: Profile B with one small to medium sized plasmid (2.3 -20kb, 42 isolates), profile C with two plasmids between 6.5 and 15kb (43 isolates) and profile D with three to nine plasmids between 3 and 25 kb (34 isolates). The different plasmid profiles did not vary according to sample matter (water, sediment, fish) or origin of the isolate (farms or sample site).

Table 4 summarizes the results of the filtermating assays, where 17 out of 40 OTC-resistant donors with plasmid profile A transferred a R-plasmid to *E. coli*. All transconjugants had received a large plasmid between 110 and 160kb.

Antibiotic resistance genes

Table 2 sums up the distribution of the identified tetracycline resistance determinants and OTC-resistant *Aeromonas* isolates. It appeared that, while 69% of the aeromonads were OTC-resistant, only 30% were carrying one or two of the five tetracycline resistance determinants included in the screening (Table 2). A total of 19 *tetA*-positive, 39 *tetE*-positive and six *tetD*-positive isolates was found. Three isolates contained two tetracycline resistance determinants: *tetA+E*, *tetA+D*, *tetE+D*, respectively. Tetracycline resistance determinants B and C were not detected.

A statistically significant increase of overall OTC-resistance levels occurred among isolates from ponds and outlets compared to inlets (37) (Table 2). However, it appeared that the respective *tetA*, *tetD* and *tetE* genes were evenly distributed among isolates from different sample sites, except among pond isolates, where more isolates with unclassified determinants were detected.

When comparing OTC-resistant motile aeromonads from the four fish farms, farm 3 isolates differed from other isolates, as only four percent of the involved tetracycline resistance

determinants were classified (χ^2 test, P< 0.005) (Table 2). Conversely, overall OTC-resistance was evenly distributed among farms.

Table 2 also shows the distribution of S/T-resistance and integrons among the aeromonads collected from water and sediments. A total of 141 isolates, including all 135 S/T- resistant isolates, contained class 1 integrons with different resistance gene inserts (Fig. 2) and an intact sul1 gene (data not shown). Six integron positive isolates without integrated gene cassettes did not express S/T-resistance phenotypically. The detected integrons were evenly distributed within all identified phenospecies (data not shown).

As S/T-resistance thus was closely correlated to the presence of class 1 integrons, the observed statistical effects were similar: Farm 1 isolates were more likely to contain integrons than isolates from other farms (logistic regression, P=0.028) (Table 2), and aeromonads from inlets of the four fish farms were less frequently integron-positive than those from ponds and outlets (logistic regression, P=0.013). One exception was farm 2, where the proportion of S/T-resistant, integron-positive *Aeromonas* isolates was higher at the inlet (44%) than in the pond (31%) or at the outlet (31%).

A schematic overview of the observed integron structures and their content of antimicrobial resistance genes is given in figure 2. Restriction enzyme profiles correlated well with the respective size of the PCR products (Fig.2), indicating that the gene content of a certain amplicon corresponded to the sequenced amplicons of the same magnitude.

A considerable number of integron positive isolates (23 out of 141) were "empty" with no gene cassettes inserted between the conserved segments of the integron. As a common feature, all integron inserts included an *ant(3'')1a* gene downstream of a trimethoprim resistance gene. Two types of dihydrofolate reductase genes were found, *dhfr1* and *dhfr2a*. The most prevalent amplicon (74 isolates) was the 1550bp PCR-product, containing *dhfrI* and *ant(3'')1a* inserts. Moreover, twenty-two 2100bp products, thirteen 1400bp products and nine 2900bp products were found among the S/T-resistant isolates. The gene inserts in the 2100bp amplicon were identical with those of the 1550bp product, with an additional downstream chloramphenicol resistance gene, *catB2*. Likewise, in the 2900bp amplicon, the two upstream gene cassettes, *dhfr2a* and *ant(3'')1a*, were the same inserts as in the 1400bp amplicons. A *catB2* cassette was identified as the last downstream gene, while the remaining insert did not yield satisfactory nucleotide sequences for identification, despite repeated purification and sequencing procedures.

As shown in table 3, there was a positive correlation between the content of large plasmids and the presence of *tetA* among OTC-resistant motile aeromonads (χ^2 test, P< 0.005). Correspondingly, *tetA* was the predominant tet-determinant detected in transconjugants (10/17), despite the comparatively low overall incidence (Tables 3+4).

By contrast, the occurrence of class 1 integrons was not related to the respective plasmid profiles, and they were just as prevalent among *Aeromonas* isolates without plasmids.

Twenty-seven out of 40 OTC-resistant donors harboured different class 1 integrons (Table 4), and they were invariably co-transferred to the *E. coli* recipient in those cases where plasmid transfer were detected (15/17).

DISCUSSION

The high OTC-resistance levels (69%) detected among the aeromonads in this study was unexpected, considering that this agent has been rarely used for therapeutic purposes in Danish aquaculture since a change in legislation in 1994. DePaola et al. (9) found similar high

proportions of OTC-resistant aeromonads from catfish and their environments (58 to 83%), where the drug was routinely used in medicated feed. Other comparable investigations of motile aeromonads from different freshwater environments report considerably lower tetracycline resistance levels (8, 11, 12, 24, 30, 43). One explanation may be that, once acquired, the resistance genes are maintained within the population, protecting the bacteria from tetracyclines produced by other members of the microflora or residues in agricultural or domestic effluents. Goni-Urriza et al. (11) detected an increase of tetracycline resistance levels of *Aeromonas* isolates from zero to 27% in a stream before and after passing a wastewater discharge point. However, this resistance appeared to be entirely chromosomally mediated (11).

More than three different tetracycline resistance determinants occurred among OTC-resistant isolates, and in a few instances more than one determinant was detected in a single strain. DePaola et al. (9) classified over 90% of tetracycline resistant *Aeromonas hydrophila* isolates from catfish farms as either *tetA*- or *tetE*-positive, and TetA, TetD and TetE are considered to be the main tetracycline resistance determinants among motile aeromonads (1, 5, 9, 10,). However, the majority of OTC-resistant isolates in this study (70%) did not belong to classes A to E (Tables 2+3). Thus, the genetic background of OTC-resistance among motile aeromonads from this habitat appeared to be rather diverse and to vary locally, possibly as a response to varying physical conditions or differences in local genetic exchange processes. Although probably not the only mechanism of horizontal resistance gene transfer, the transfer of R-plasmids is thought to play a major role in the dissemination of OTC-resistance in the fish-farming environment (5, 32, 41, 42, 45). In our study, there was a positive correlation between OTC-resistant motile aeromonads harbouring large plasmids and the presence of *tetA*. Moreover, the majority of successfully transferred R-plasmids carried the *tetA* resistance gene. This contrasts DePaola's findings, where most donors in successful matings had unidentified tetracycline resistance determinants (9, 10).

Human as well as environmental *Aeromonas* isolates do often contain conjugative R-plasmids, some of which have been assigned to incompatibility groups C and U (15, 32). Both groups have wide host ranges, yet IncU plasmids have only been detected occasionally in genera other than *Aeromonas* (15, 46). During the course of this study, we successfully transferred OTC-resistance plasmids to susceptible *Aeromonas* field isolates in addition to the *E. coli* strain (data not shown). In another set of experiments, the role of conjugation under simulated natural conditions was investigated, including environmental donors and recipients (7).

Rhodes et al. (32) reported that IncU OTC-resistance plasmids in mesophilic aeromonads from hospital effluents and fish farms are closely related to IncU OTC-resistance plasmids isolated from the fish pathogen *Aeromonas salmonicida* and a human *E. coli* strain. The predominant tet-determinant was *tetA*, and the presence of a complete or truncated form of tetracycline resistance transposon Tn1721 was demonstrated on several of the R-plasmids, thus proposing yet another mechanism of horizontal dissemination of antimicrobial resistance in these settings (32, 38, 45).

The present work demonstrated the common occurrence of class 1 integrons and their resistance gene cassettes on OTC-resistance plasmids. Still, there was no evidence of a correlation between the occurrence of integrons and a certain plasmid type or tet-determinant. Conversely, there was a close association of class 1 integrons and S/T-resistance, as all S/T-resistant isolates contained an integron with a *sul1* gene within the 3'conserved and a dihydrofolate reductase gene cassette insert. Aeromonads from the inlets compared to the ponds and outlets of the fish farms were less likely to be OTC-, OXA- and S/T-resistant (37), and consequently integron-positive. Furthermore, *Aeromonas* isolates from farm 1 were significantly more likely to be S/T-resistant and integron-positive than isolates from the farms further downstream, perhaps due to a more frequent use of

the drug at this farm during the trial period (37). It may be speculated, that the distribution of these genetic elements is enhanced by the frequent use of potentiated sulphonamides in the fishfarming environment, including occasional additional resistance gene cassettes like *ant(3'')1a* or *catB2*. If such integrons are mobilized onto R-plasmids, indirect selection could contribute to the maintenance of tetracycline resistance genes within the population.

Only few researchers so far have addressed the incidence and spread of class 1 integrons and integron-associated resistance genes in environmental microorganisms (29, 33, 45). Even in clinical settings, the epidemiology of integrons and gene cassettes is not resolved (23, 31, 34, 40, 44). Rosser and al. (33) detected the incidence of class 1 integrons to be 3.6% among Gram-negative bacteria from an estuarine environment. Unlike in this study, almost half of the integrons lacked a *sul1* gene, and about the same proportion did not have any genes inserted into the variable region. The authors propose that gene cassettes are excised from the structure in the absence of antibiotic selective pressure, or that "empty" integrons represent ancestral elements, which have not yet acquired gene cassette inserts.

The abundance of class 1 integrons and the inserted *dhfr* genes among the motile aeromonads correlates with transient selective pressures exerted by the administration of combined sulphonamide/ trimethoprim drug in freshwater fish farms. Aminoglycosides and chloramphenicol, on the other hand, are not used in Danish aquaculture. Possibly, aquatic motile aeromonads have acquired the respective gene cassettes by interacting with other microorganisms in soil or domestic effluents (33, 45). Aminoglycoside resistance gene cassettes may also be more stably integrated within integrons, explaining their frequent occurrence and persistence in many bacterial species and habitats (34).

In conclusion, our results point towards a significant effect of aquaculture on the motile aromonads in and around the investigated fish farms, leading to an increase of oxytetracycline-, sulphadiazine/trimethoprim- and oxolinic acid resistance levels within this group. High levels of multiresistance (48%) indicated that horizontal spread of resistance genes has contributed to the evolvement of in particular OTC-S/T-resistance phenotypes (37), and the finding of class 1 integrons in 45% of the isolates further supported this hypothesis. The diversity of the isolates and characterized genes does not support the clonal spread of antibiotic resistant aeromonads in and around the fish farms.

Conjugation assays demonstrated that different types of class 1 integrons were co-transferred to *E. coli*- recipients on OTC- resistance plasmids. However, it was evident that other genes and transfer mechanisms besides conjugation are probably involved, which should be further investigated in the future. The significance of antibiotic resistant environmental bacteria has been much disputed (22, 32, 33, 45). Although ubiquitous in freshwater, motile *Aeromonas* species are not commonly retrieved from human or animal disease in temperate climate zones (16). However, extensive antimicrobial resistance within this group might provide a pool of resistance genes capable of transfer to other waterborne bacteria or fish pathogens. A better understanding of such processes in natural environments is crucial in order to assess the risk of antibiotic resistance among ubiquitous aquatic bacteria such as the motile aeromonads.

ACKNOWLEDGEMENTS

This study was supported by the Danish Ministry of Food, Agriculture and Fisheries. We thank Farah Bahrani for excellent technical assistance, Frank M. Aarestrup, Lars Bogø Jensen and Dorthe Sandvang at the Danish Veterinary Laboratory, Copenhagen, for various aspects of the initial sequencing work. We are also grateful to Professor Ib Skovgaard and Associate Professor Bo M. Bibby of the Department of Mathematics and Physics, The Royal Veterinary and Agricultural University, for their assistance with the statistical analysis.

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Table 1. PCR primer sequences, targets and annealing sites

Primer	Nucleotide sequence (5'- 3')	Target	Annealing sites	Accession no. (GenBank)
TetA F	GTA ATT CTG AGC ACT GTC GC	*tetA*	4925-4942	X00006
TetA R	CTG CCT GGA CAA CAT TGC TT		complement to 8340-8323	X00006
TetB F	CTC AGT ATT CCA AGC CTT TG	*tetB*	2378-2397	J01830
TetB R	CTA AGC ACT TGT CTC CTG TT		complement to 2813-2794	J01830
TetC F	TCT AAC AAT GCG CTC ATC GT	*tetC*	92-111	J01749
TetC R	GGT TGA AGG CTC TCA AGG GC		complement to 680-661	J01749
TetD F	ATT ACA CTG CTG GAC GCG AT	*tetD*	1551-1570	X65876
TetD R	CTG ATC AGC AGA CAG ATT GC		complement to 2674-2654	X65876
TetE F	GTG ATG ATG GCA CTG GTC AT	*tetE*	32-52	L06940
TetE R	CTC TGC TGT ACA TCG CTC TT		complement to 1231-1212	L06940
int 1F	GGC ATC CAA GCA GCA AGC	*intI*	4925-4942	U12338
int 1R	TAG TCC AGT TCA GAC GAA	*qacE*Δ	complement to 8340-8323	U12338
qacF	ATC GCA ATA GTT GGC GAA GT	*qacE*Δ	8417-8436	U12338
sul R	GCA AGG CGG AAA CCC GCG CC	s*ul1*	complement to 9214-9195	U12338
dhfr F	CTG ATA TTC CAT GGA GTG CCA	*dhfrI*	140-160	AF203818
dhfr R	CGT TGC TGC CAC TTG TTA ACC		complement to 553-573	AF203818
ant F	GCC TGA AGC CAC ACA GTG ATA	*ant(3")-1a*	772-791	AF203818
ant R	CTA CCT TGG TGA TCT CGC CTT		complement to 1411-1391	AF203818

Table 2. Incidence and distribution of oxytetracycline (OTC) and sulphadiazine/trimethoprim (S/T) resistance (37) and the corresponding fractions of identified tetracycline resistance determinants (Tet) and class 1 integrons (Int) among motile aeromonads isolated from four Danish rainbow trout farms. The comparison of different sampling sites (inlets, ponds and outlets at the four farms) was based on *Aeromonas* isolates from water and sediment samples, while the comparison of fish farms also included fish isolates.

	(no. of isolates)	%			
		OTC	Tet	S/T	Int
Sampling site	Inlet (53)	49 [a]	17	19 [a]	25 [a]
(n=250)	Pond (92)	71	33	49	47
	Outlet (105)	77	14	47	45
	Avg	66	22	38	39
Fish farm	Farm 1 (69)	65	17	61 [b]	67 [b]
(n=313)	Farm 2 (98)	75	30	42	43
	Farm 3 (53)	68	4 [c]	25	27
	Farm 4 (93)	67	29	42	37
	Avg	69	20	43	44

[a] Statistically significant lower fraction compared to other locations at the farms.
[b] Statistically significant higher proportion compared to other farms.
[c] Statistically significant lower fraction compared to other farms.

Table 3. Correlation of plasmid profiles and tetracycline resistance determinants (Tet) among 216 oxytetracycline (OTC)-resistant and 97 OTC-sensitive *Aeromonas* isolates and the association of tetracycline resistance determinants with self-transferable plasmids, as established in filtermating assays with *E. coli* recipients.

	No. of isolates				
	OTC-resistant isolates				Sensitive isolates
		TetA	TetD	TetE	NC[b]	
Plasmid profile [a]						
	A	13	2	5	20	10
	B, C, D	1	3	18	68	29
	no plasmids	5	1	16	64	58
	Total	19	6	39	152	97
Donors		13	2	5	20	-
Transconjugants		11	1	0	5	-

[a] Profile A: one or several large plamids (>30kb); profiles B, C, D: small to medium sized plasmids (2.3 -25kb).

[b] NC, not classified tetracycline resistance determinant.

Table 4. Characterization of 40 oxytetracycline-resistant, motile *Aeromonas* isolates with large (>30kb) plasmids from a freshwater fish farming environment, including presumptive biochemical identification (ID) and detection of antibiotic resistance genes. Tc + indicates the detection of transconjugants in filtermating assays, where A*eromonas* donors transferred both their respective tetracycline (Tet) resistance determinant and class 1 integron on R-plasmids to *E. coli* recipients.

Isolate no.	Date	Source	ID	Resistance phenotype	Tet	Class 1 integrons/ gene inserts	Tc
1-75	11-97	Water/ inlet	*A. hydrophila*	OTC, S/T	A	*dhfr2a-ant(3")1a*	+
1-78	11-97	Water/ pond	*A. hydrophila*	OTC, OXA, S/T	E	ND [c]	
1-163	4-98	Rainbow trout	*A. hydrophila*	OTC, S/T	A	*dhfr1-ant(3")1a-catB2*	
1-231	6-98	Sediment/ outlet	*A. hydrophila*	OTC, OXA, S/T	D	ND	+
1-250	7-98	Rainbow trout	NI [a]	OTC, S/T	NC[b]	*dhfr1-ant(3")1a*	+
1-318	10-98	Sediment/ pond	*A. hydrophila*	OTC	E	ND	
1-337	10-98	Water/ pond	*A. veronii* bv *sob.*	OTC, S/T	A	*dhfr1-ant(3")1a-orf-catB2*	+
1-358	10-98	Sediment/ outlet	*A. veronii* bv *sob.*	OTC, S/T	A	*dhfr1-ant(3")1a-orf-catB2*	+
1-372	12-98	Sediment/ outlet	*A. hydrophila*	OTC, OXA, S/T	NC	ND	
2-41	10-97	Rainbow trout	*A. bestiarum*	OTC, S/T	NC	*dhfr1-ant(3")1a*	+
2-62	11-97	Sediment/ outlet	*A. hydrophila*	OTC, S/T	A	*dhfr2a-ant(3")1a*	+
2-85	1-98	Water/ pond	*A. hydrophila*	OTC, S/T	A	*dhfr1-ant(3")1a-orf-catB2*	+
2-155	4-98	Water/ pond	*A. hydrophila*	OTC, OXA	NC	ND	+
2-189	6-98	Water/ outlet	NI	OTC, S/T	NC	*dhfr1-ant(3")1a*	+
2-197	6-98	Rainbow trout	NI	OTC, S/T	NC	*dhfr1-ant(3")1a-catB2*	
2-219	6-98	Rainbow trout	*A. hydrophila*	OTC, S/T	NC	ND	
2-280	9-98	Sediment/ pond	*A. hydrophila*	OTC, S/T	A	*dhfr1-ant(3")1a-catB2*	+
2-282	9-98	Sediment/ outlet	*A. hydrophila*	OTC	NC	ND	
2-321	9-98	Sediment/ outlet	*A. hydrophila*	OTC, OXA	NC	ND	
2-333	10-98	Sediment/ outlet	NI	OTC, OXA	NC	ND	
2-410	2-99	Sediment/ pond	*A. bestiarum*	OTC, S/T	A	*dhfr1-ant(3")1a*	
3-30	10-97	Sediment/ outlet	*A. hydrophila*	OTC, S/T	NC	*dhfr1-ant(3")1a*	
3-130	3-98	Sediment/ outlet	*A. hydrophila*	OTC, OXA, S/T	NC	ND	
3-135	4-98	Water/ inlet	*A. hydrophila*	OTC	NC	ND	
3-217	7-98	Water/ inlet	*A. hydrophila*	OTC	NC	ND	
3-247	9-98	Sediment/ outlet	NI	OTC	NC	ND	
3-372	2-99	Water/ pond	*A. hydrophila*	OTC, OXA, S/T	NC	*dhfr2a-ant(3")1a*	+
4-90	11-97	Rainbow trout	*A. hydrophila*	OTC, S/T	E	*dhfr1-ant(3")1a*	
4-97	1-98	Water/ outlet	*A. hydrophila*	OTC, S/T	E	*dhfr1-ant(3")1a-catB2*	
4-206	6-98	Sediment/ pond	*A. hydrophila*	OTC, S/T	AE	*dhfr1-ant(3")1a-catB2*	
4-221	6-98	Rainbow trout	*A. veronii* bv *sob.*	OTC, S/T	A	*dhfr1-ant(3")1a-orf-catB2*	+
4-229	7-98	Rainbow trout	NI	OTC, OXA, S/T	A	*dhfr1-ant(3")1a*	+
4-241	7-98	Rainbow trout	*A. veronii* bv *sob.*	OTC, S/T	NC	*dhfr1-ant(3")1a-orf-catB2*	
4-280	9-98	Rainbow trout	*A. bestiarum*	OTC, OXA, S/T	NC	*dhfr1-ant(3")1a-orf-catB2*	+
4-302	9-98	Sediment/ inlet	*A. hydrophila*	OTC	NC	No inserts	
4-307	9-98	Rainbow trout	*A. veronii* bv *ver.*	OTC, OXA, S/T	A	*dhfr1-ant(3")1a-orf-catB2*	+
4-348	9-98	Rainbow trout	*A. hydrophila*	OTC, S/T	NC	*dhfr1-ant(3")1a*	

4-353	9-98	Sediment/ inlet	*A. hydrophila*	OTC	NC	No inserts	
4-354	9-98	Sediment/ inlet	*A. bestiarum*	OTC	E	No inserts	
4-440	2-99	Sediment/ pond	*A. hydrophila*	OTC, S/T	A	*dhfr1-ant(3")1a-orf-catB2*	+

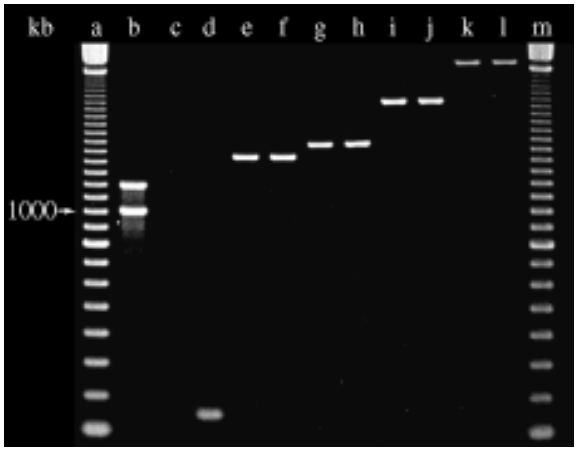
OTC, oxytetracycline; OXA, oxolinic acid; S/T, sulfadiazine/ trimethoprim.

[a] NI, no reliable identification of *Aeromonas* phenospecies.

[b] NC, not classified tetracycline resistance determinant.

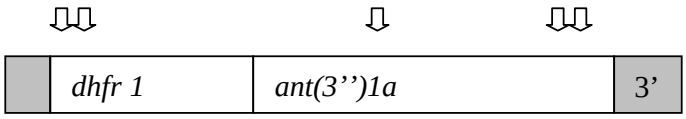
[c] ND, not detected.

Fig. 1 Different sizes of PCR-products obtained with a primerpair targeting the conserved segments of class 1 integrons in motile aeromonads. Class 1 integrons were- if present- invariably co-transferred to *E. coli* on oxytetracycline resistance plasmids.

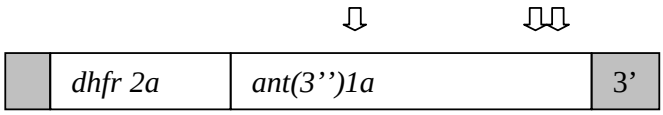


Lanes a and m, 100bp DNA marker; lane b, positive control *Salmonella typhimurium* DT 104 with 1000- and 1200bp amplicons; lane c, neg. control; lane d, “empty” integron with no gene inserts between conserved ends; lanes e and f, isolate 1-75 and the corresponding *E. coli* transconjugant with 1400bp amplicons; lanes g and h, isolate 4-229 and transconjugant with 1550bp amplicons; lanes i and j, isolate 2-280 and transconjugant with 2100bp PCR-products; lanes k and l, isolate 4-228 and transconjugant, with 2900bp products.

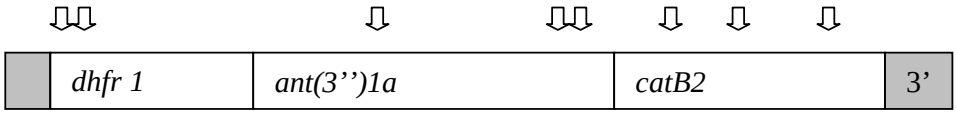
Fig. 2. Schematic view of MboII restriction sites (⇩) used for comparison of gene cassette content of different sizes of amplicons obtained with primers targeting the conserved segments (shaded areas) of class1 integrons. Out of 141 integron-positive isolates, 74 belonged to profile I, 13 to profile II, 22 to profile III and nine to profile IV. Twenty-three isolates had "empty" integrons with no genes inserted into the variable region. UI, unidentified gene insert.



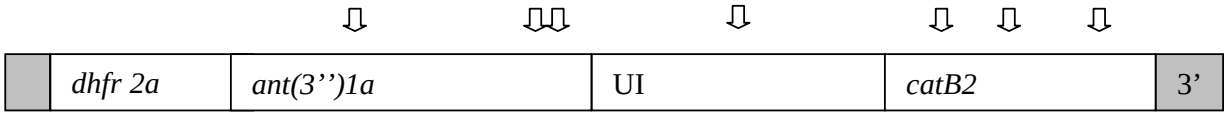
profile I (1550bp)



profile II (1400bp)



profile III (2100bp)



profile IV (2900bp)

Conjugal transfer of oxytetracycline resistance in vitro and in membrane filter chambers: transfer between environmental aeromonads, fish pathogenic bacteria and *Escherichia coli*

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Running title: Transfer of OTC-resistance in membrane filter chambers

ABSTRACT

The ability of environmental motile *Aeromonas* species and fish pathogenic bacteria to transfer oxytetracycline resistance to various bacterial species was assessed in vitro and in situ. Oxytetracycline resistant motile aeromonads and *Aeromonas salmonicida* with large plasmids were used as donors and a rifampicin resistant *Escherichia coli* as recipient. In addition, the fish pathogens *Yersinia ruckeri* and *Flavobacterium psychrophilum* were used as recipients as well as a *Pseudomonas putida* strain and motile aeromonads originating from aquatic environments.

Transfers were seen in both laboratory and environmental settings. In vitro transfers using filter mating yielded transfer frequencies from 1.4×10^{-9} to 6.8×10^{-5} transconjugants per recipient. Transfer to *E. coli* was seen from 40% of motile *Aeromonas* spp. Tetracycline determinants A, D and unidentified tetracycline determinants were transferred usually located on plasmids of approximately 150 kb size. Transfer was detected in microcosms both in a simple laboratory set-up with sediment and water in vials and in membrane filter chambers in aquaria and in situ at the outlet of a freshwater fish farm.

Two motile *Aeromonas* isolates were used as donors for in situ experiments and transfer frequencies ranged from 5.9×10^{-8} to 5.5×10^{-4}. Transfer was seen to *Y. ruckeri* and motile *Aeromonas* spp. but no transfer to *F. psychrophilum* was detected. The fish pathogen *A. salmonicida* transferred R-plasmids to *E. coli* but no transfer was detected to a motile *Aeromonas* spp. in membrane filter chambers.

INTRODUCTION

Antimicrobial resistance has been a major concern especially in hospitals during the last two decades (12). Indiscriminate use of antimicrobial agents in society and hospitals has been blamed, but also use in animal production and subsequent transfer of resistant bacteria or resistance genes to human pathogens is suspected to compromise the clinical efficacy of some broad-spectrum antimicrobial agents (17,33,46).

Transfer of antimicrobial resistance has been assessed widely in vitro under more or less natural conditions (5,13,19,21,23,29,36,40) and to a lesser extend in situ (2,7,11,16,22,25,32). These experiments are often carried out with few strains of clinical origin. In contrast, this study uses bacterial isolates from a field trial (43). Bacteria isolated from water, sediment and fish are used in transfer experiments under various conditions in vitro and in situ.

Motile mesophilic *Aeromonas* spp. are ubiquitous bacteria in natural aquatic environments (20,35,38,47), where some species are known to cause diseases in fish and other poikilotherms (6) and others are regarded as opportunistic human pathogens (27,31). In Denmark, motile aeromonads rarely cause disease neither in humans nor in farmed animals. Antimicrobial resistance and R-plasmids are apparently common among *Aeromonas* spp. (20,24,35,38). Likewise, high numbers of oxytetracycline (OTC) resistant aeromonads were found during the field trial at fresh water fish farms (42) and thus the objective of subsequent experiments was to investigate if and to what extend these isolates transferred their resistance. Transfer was shown from 16 out of 40 (40%) motile *Aeromonas* spp. with large plasmids and oxytetracycline resistance (42). The transfer frequencies are presented in Table 1.

Two of the 16 isolates that transferred R-plasmids to *E. coli* were used in transfer experiments with other recipients than *E. coli* and in the experimental settings more akin to natural environmental conditions to demonstrate transfer of naturally occurring plasmids containing tetracycline resistance determinants to a laboratory *Escherichia coli* strain, to other environmental isolates (*Aeromonas* spp.) and fish pathogens (*Flavobacterium psychrophilum*, *Yersinia ruckeri*).

A stepwise approach was followed where transfer initially was tested by laboratory filter matings under what was believed to be optimum conditions with high bacterial load, extensive surface area and nutrient rich environment at 20°C (19). To mimic natural conditions the next set-up consisted of sterile water and sediment in a test tube and afterwards transfer experiments were carried out using membrane filter chambers both in a laboratory aquarium containing tap water and finally, in the stream from which the isolates originated.

MATERIALS AND METHODS

Bacterial isolates

Putative donors were selected from our collection of motile aeromonads (42,43). Isolates that were both resistant to oxytetracycline (MIC > 4 mg/L), contained large (>25 kb) plasmid(s) and had shown transfer in laboratory filter matings with *E. coli* (CSH26Rf) as recipient. Initially the isolates 1-75, 1-163, 2-62 and 4-221 were used but in the membrane filter chamber set-up only 1-75 and 2-62 were used. The isolates used in succeeding experiments: 1-75 and 2-62 showed transfer frequencies in filter matings of 3.4×10^{-5}, 1.0×10^{-6} and 1.1×10^{-5}, 2.6×10^{-6} respectively (Table 1).

In addition, oxytetracycline resistant *A. salmonicida* isolates were used as donors, as presented in a previous article (44), and two of these isolates (950704-2/1, 718) were used in further experiments.

A rifampicin resistant *E. coli* strain (CSH26Rf) without plasmids was used as recipient in all initial experiments. Furthermore, transfer experiments with selected isolates of motile *Aeromonas* spp., *F. psychrophilum* and *Y. ruckeri* as recipients were carried out (Table 2). Motile *Aeromonas* spp. and *F. psychrophilum* isolates to be used as recipients were sensitive to oxytetracycline but carried resistance to oxolinic acid, whereas selected *Y. ruckeri* carried florfenicol resistance to be used in counterselection. As counterselection with oxolinic acid was unsatisfactory, an *Aeromonas* isolate (4-251) was plated on a rifampicin gradient plate (18) to obtain a resistant mutant for use as recipient in transfer experiments (4-251Rf).

None of the 134 *Y. ruckeri* isolates previously described (43) carried oxytetracycline resistance. Consequently, transfer experiments with this species as donor could not be performed and the isolates were not screened for tetracycline resistance determinants.

Of the 89 *F. psychrophilum* isolates previously described (43), 63 were resistant to oxytetracycline, but none contained large plasmids and thus no transfer experiments were conducted with this species as donor.

Susceptibility testing

Isolates were tested in a standardized agar dilution assay (37) in order to determine their MIC-values towards oxytetracycline and other antimicrobial agents used in Danish fresh water aquaculture as described earlier (9,43).

Plasmidprofiling

All aeromonads and *E. coli* transconjugants were screened for their plasmid content, applying the alkaline lysis method described by Kado and Liu (28), followed by agarose gel electrophoresis.

Detection of Tet-determinants

A multiplex PCR-assay was used according to Guardabassi et al. (23), to test all OTC-resistant isolates for tet-determinants A-E. See Schmidt et al. (42) for further details. The *F. psychrophilum* isolates were screened for tetracycline resistance determinants A, B, C, D, E, and ten isolates were screened for K, L, M, O and S, determinants mainly found in Gram positives.

Conjugal gene transfer

Filtermating assay

Donor and recipient were each cultured in veal infusion broth (Difco Laboratories, Detroit, Mich., USA) at 20°C with shaking overnight and subsequently adjusted to OD_{600}=0.5 with fresh veal infusion broth. *F. psychrophilum* was grown in tryptone yeast extract salts (TYES) broth (26) for two days at 15°C. A sterile 0.2µm nitrocellulose filter (Sartorius AG, Goettingen, Germany) was placed on a veal infusion agar plate (Difco), 50µl of each culture were mixed on the filter and incubated at 20°C overnight. Experiments with *F. psychrophilum* were done using 1.1 % TYES

agar (26) and incubation at 15°C. Cells were washed off the filter by vortexing in 10 ml sterile 0.9% NaCl solution, and appropriate tenfold dilutions were prepared. From each dilution, three 25µl droplets were applied to selective agar plates, containing 20 µg ml^{-1} oxytetracycline or 100 µg ml^{-1} rifampicin (Bie&Berntsen, Rødovre, Denmark) and 100 µl were spread plated on double selective plates containing both antimicrobial agents. Donor and recipient were also plated on the double selective plates for mutant detection. All assays were run in duplicate. Transfer frequencies were calculated as the mean number of transconjugants per recipient (Table 1).

Presumptive transconjugants were isolated in pure culture and tested for motility, catalase, cytochrome oxidase, arginine dihydrolase, ornithine decarboxylase, lactose and indol to assure that no rifampicin-resistant mutants of the original donor or possible contaminants were selected. Transconjugants were screened for the presence of plasmids as described above.

Experiments with motile *Aeromonas* spp. and *F. psychrophilum* isolates as recipients were carried out with agar plates containing 8 µg ml^{-1} oxolinic acid instead of rifampicin, and with *Y. ruckeri* as recipient rifampicin was substituted with 4 µg ml^{-1} florfenicol.

Simple microcosm assay

Overnight cultures of donor and recipient (50 µl of each) were transferred to a test tube containing approximately one gram of sterile sediment and one ml of sterile filtered stream water. The test tube was whirl mixed and incubated at 20°C for 18 to 20 hours. Appropriate tenfold dilutions were plated as described above.

Membrane filter chamber assay

Chambers (Montana State University, College of Engineering Technical Services, USA) (34) were assembled using 0.22 µm pores size Durapore membrane filters (Millipore, Cork, Ireland) with approximately one gram of sediment inside and autoclaved. The volume of the chamber was 100 ml. The chamber was immersed in water, hanging from an overhead support and keeping the pipe stubs above water. Donor and recipient were grown in 20 ml veal infusion broth (Difco) at 20°C with shaking overnight. Ten ml of each broth culture were transferred via a marked stub to the chambers using plastic syringes. Samples were removed from the other stub using a five ml syringe after 2, (4,) 6, 24 (and 48) hours, respectively. Before each sampling the content of the chamber was mixed by aspirating a few times. Approximately 2 ml were removed and plating was performed as described above at each sampling time. Aquarium experiments were performed at approximately 11°C and 20°C whereas the water temperatures during the two field trials were approximately 11°C (12.0°C and 10.7°C on the 25/7-00 and 17/10-00, respectively). The field trials were both done at the outlet of a fish farm located at the stream where the environmental isolates were collected previous years. No antimicrobial agents were used at this fish farm during or at least a month before the experiments and oxytetracycline has not been used for the last fifteen years or more. Chambers were located in the large sedimentation pond close to the bank in calm, flowing water. The previous year this fish farm produced 210 t of fish and used 184 t of fish feed. All discharge requirements of the authorities were satisfied.

All experiments were repeated on different days.

RESULTS

Using three *Y. ruckeri* isolates as recipients in filter mating experiments yielded transconjugants with only 4-49 from both 1-75 and 2-62 (Table 2). Transfer frequencies were 2.1 x 10^{-5} and 1.7 x 10^{-7} respectively.

Only two of the 63 resistant *F. psychrophilum* isolates were tested positive for Tet C. The genetic background for the resistance in the remaining isolates is not known. No transfer was seen when three *F. psychrophilum* isolates were used as recipients in filter matings with 1-75 and 2-62 as putative donors (Table 2).

Intrageneric transfers to other motile *Aeromonas* spp. were successful in five cases with transfer frequencies of 1.6 x 10^{-6} to 1.9 x 10^{-3} (Table 2). In two cases transfer between motile *Aeromonas* spp. yielded different transconjugants with variable numbers of plasmids transferred. Transfer from 2-62 to 1-126 yielded transconjugants with three different plasmid profiles containing, respectively, one large (160 kb), the large and one small (7 kb) plasmid and finally all eight plasmids were transferred. Likewise, different numbers of plasmids were transferred from 1-75 to 1-181. One transconjugant received only the large (160 kb) plasmid while the other received a small plasmid as well in addition to the small plasmid carried originally by 1-181. In general more than one transconjugant were picked but this transfer heterogeneity was only seen in these two cases.

Transfer from motile *Aeromonas* spp. to *E. coli* in simple microcosms with water and sediment yielded frequencies in the range 2.5 x 10^{-9} to 2.6 x 10^{-7} (Table 3). Laboratory experiments with membrane filter chambers showed rather variable transfer frequencies from 2.2 x 10^{-11} to 5.1 x 10^{-4} (Table 4). Transfers were seen at both 20°C and 11°C.

In situ transfer frequencies ranged from 5.9 x 10^{-8} to 5.5 x 10^{-4}. Transfer was detected from 1-75 and 2-62 to *E. coli* and from 1-75 to the motile *Aeromonas* 4-251 but not to the *Y. ruckeri* tested (Table 4).

Frequencies were apparently reflecting the changes in recipient number, as the number of transconjugants in several cases were stabilized rather quickly (Fig. 1), and the fluctuations in frequencies are a result of changes in number of recipients. This is not the case in the transfer experiments from 1-75 to *E. coli* in the stream where the number of transconjugants apparently did not reach a steady level within the period of sampling (Fig. 1).

In one case, transconjugants were not seen at any of the first sampling times but only after 24 hours (Table 4).

In general, *E. coli* survived fairly well for up to two days in the membrane filter chambers. The donors 1-75, 2-62 and 718 also survived well. After an initial decline the isolate 1-75 was even able to grow at 11°C in chambers submersed in the stream. The exception was 4-251 used as recipient, which disappeared totally after 24 hours (Fig. 1)

Results of mating experiments with *A. salmonicida* as donor and *E. coli* as recipient were presented in a previous article (44). Transfer was only seen from three of 23 Danish isolates. In contrast transfer occurred from 16 of the 17 foreign isolates.

Further transfer experiments with one of the positive Danish isolates and the atypical *A. salmonicida* 718 isolate yielded transfer of resistance from the Danish strain 950704-2/1 to *E. coli* in situ with a frequency of 1.3 x 10^{-7} (Table 4). The plasmid transferred was approximately 150 kb of size. The atypical 718 transferred a 32 kb plasmid to *E. coli* in aquarium experiments with membrane filter chambers at both 20°C and 11°C. No transfer to the motile *Aeromonas* 4-251 was detected from either of the *A. salmonicida* isolates although this was seen after filter matings (data not shown).

DISCUSSION

Most of the plasmids transferred had a size of approximately 150 kb, but also slightly larger plasmids were transferred and in one case a smaller plasmid around 120 kb transferred along with small plasmids of 3, 5 and 10 kb (Table 1). Plasmids of approximately 150 kb were common among motile *Aeromonas* spp. with large plasmids, also among strains where no transfer was detected (Table 1). Various tetracycline determinants and integrons with different gene cassettes (42) were located on plasmids of similar sizes, and thus nothing indicated that it was the same plasmid that was responsible for all conjugations.

The transfer of resistance from 40 % of the tested motile *Aeromonas* spp. was a rather high success rate compared to the results of Ansary et al. (1992), who examined 34 *A. hydrophila* strains from fish. Fifteen strains carried plasmids, but no transfer of resistance plasmids was detected to *E. coli* or *A. hydrophila* (4). Nevertheless, transfer of resistance plasmids from motile Aeromonas spp. has been demonstrated with 22 *Y. ruckeri* strains as recipients by Klein et al. (30), who reported no transfer from nine *A. hydrophila* strains and transfer from 5 *A. sobria* strains out of 25 isolates from fish.

Altherr & Kasweck (2) isolated coliforms from sewage and transferred tetracycline resistance to *E. coli*. Transfer frequencies in vitro were 4.4×10^{-5} to 1.6×10^{-4} and 1.0×10^{-6} to 3.2×10^{-5} in membrane diffusion chambers (2). The higher rate of transfer compared to our study, might be a result of intrageneric transfer in comparison to the intergeneric transfer from *Aeromonas* spp. to *E. coli*. De Grandis & Stevenson (13) found higher transfer frequencies in transfers between *Y. ruckeri* strains than from *Yersinia* to *E. coli* (13). It remains uncertain if a laboratory strain as recipient is more efficient than environmental or fish pathogenic bacteria or if only the phylogenetic relatedness of donor and recipient matters. The transfer frequencies from motile *Aeromonas* spp. to *E. coli* in this study ranged from 7.0×10^{-10} to 6.8×10^{-5} transconjugants per recipient (Table 1).

Different tetracycline determinants were transferred. Ten cases of Tet A (63%), one Tet D (6%) and 5 unidentified tetracycline determinants (31%). A statistically significant higher fraction of Tet A positive strains conferred resistance by conjugation than isolates with unidentified determinants. None of the six Tet E determinants were transferred in this study.

The Tet D determinant transferred from 1-231 conferred the high-level resistance present in the donor to the transconjugant with a MIC_{OTC} of 512 mg l^{-1}, whereas other donors and transconjugants had MIC values in the range from 16 to 64 mg l^{-1}. The unidentified tetracycline determinants, which were transferred in our study, could be determinants previously described and not tested for here, or entirely new determinants. Clinical bacterial isolates mainly contain known Tet determinants (1,23,39,44) whereas a large proportion of environmental isolates contain non-typeable determinants (3,14,23,42). These findings suggest that although gene transfer might occur between clinical and environmental bacteria, there are some limitations in regard to transfer between compartments. This is further strengthened by the differences in plasmid sizes and carried integrons found on the plasmids examined (42), which shows no indication towards clonal spread of antimicrobial resistance genes. This is in contrast to the belief of Rhodes et al. (41), who suggested that the aquaculture and human compartments of the environment behave as a single interactive compartment with clonal spread of Tet A on the transposons Tn1721 (41).

Host range of the resistance plasmids could be important both in relation to species of the recipient (incompatibility) and the conditions under which transfer experiments are carried out. Transfer frequencies of i.e. IncP plasmids with short and rigid pili are generally higher on solid surfaces than in liquids, where plasmids encoding flexible pili are more efficiently transferred

(16). Hedges et al. (24) reported the compatibility specificity of resistance plasmids from *A. hydrophila* and *A. salmonicida.* Most bacterial species can carry several different plasmid groups and *Aeromonas* supported at least three: group U and group C, which both have a wide host range, and another unidentified group that was unstable in *E. coli* (24). This instability was not observed with the transconjugants examined in our study.

Transfer in simple microcosms yielded lower transfer frequencies than both filter matings and most experiments in membrane filter chambers. A lower surface area in this set-up might be the explanation.

The membrane filter chambers were well suited for the experiments – easy to use and no contamination occurred in control chambers placed in the stream containing the donor or recipient alone. It shall be stressed that even if the conditions in the chambers in some regards mimic the surrounding environment, two obvious differences might be important: firstly, the bacterial load/density of the involved donor and recipient strain are higher than expected in most environmental niches, and secondly, there is no direct interaction with the diverse and complex bacterial community found outside the chambers in water, sediment and biofilm where many different species interact. Generally, the fresh water environments examined are depleted in nutrients and the temperature is below those optimal for exponential growth – even for the bacteria isolated from these environments. But nutrient rich conditions with high bacterial density can be found in several niches that could act as hot spots for gene transfer. Some of the possible hot spots have been described in the literature, where higher transfer efficacy was seen in the residuesphere (15,45) and the gut of microarthropods (25) and probably this is valid in the gut of other animals e.g. fish.

Nevertheless, plasmid transfer can take place under oligotrophic conditions as shown by Goodman et al. (21) who transferred the broad-range plasmid RP1 between starved marine *Vibrio* S14 and *E. coli* (21). Likewise, Chandrasekaran et al. (10) detected transfer of a multiresistance plasmid from *Pseudomonas fluorescens* to marine bacteria even after the donor cells entered the viable but nonculturable state (10). The bacteria used for transfer experiments in membrane filter chambers in the present study were not starved before they entered the chambers but as the semipermeable membrane allowed diffusion of nutrients the conditions inside the chamber quickly became oligotrophic. Inclusion of sediment inside the chambers increased the surface area available for conjugation and mimicked the stream bottom sediment where high concentrations of indigenous bacteria occur. To our knowledge this is the first time transfer of resistance plasmids from motile *Aeromonas* spp. have been examined under conditions mimicking the natural environment. Most similar experiments have been conducted with various *Pseudomonas* strains or *E. coli* (16).

Transfer of resistance plasmids from motile *Aeromonas* spp. to fish pathogenic bacteria was shown from both 1-75 and 2-62 to one *Y. ruckeri* isolate whereas no transfer was detected to two other *Y. ruckeri* isolates (Table 2). Klein et al. (30) reported that 70% of 22 *Y. ruckeri* strains showed recipient capacity but no single donor transferred plasmids to more than a maximum of 9 *Y. ruckeri* strains (30). In the present study no transfer was detected to *F. psychrophilum* or *P. putida* from isolates capable of transferring plasmids to *E. coli* (Table 2). This supports that not all bacterial species will be receptive to plasmids from e.g. motile aeromonads. Likewise, not all members within a species show equal recipient capacity.

The lack of transfer from *A. salmonicida* to the genetically related motile *Aeromonas* spp. in membrane filter chambers might be caused by the disappearance of the recipient 4-251 during the experiment as seen with 1-75 as donor (Figure 1). Transfer was detected to motile *Aeromonas*

spp. after filter matings and Klein et al. (30) transferred resistance plasmids from *A. salmonicida* to *Y. ruckeri*.

The results presented support previous findings, which indicate, that transfer of plasmids carrying oxytetracycline resistance determinants is dependent on donor, recipient, time span and physiochemical conditions at the place of action (16). As pointed out by i.e. Baquero et al. (8), the generation of antimicrobial resistance is a multifactorial process where the role of selective pressure and bacterial diversity not always are well defined, even if it seems clear that antimicrobial residues can select for antimicrobial resistance in aquatic bacteria. The transfer of oxytetracycline resistance both in filter matings and in membrane filter chambers occurred without any obvious selection pressure in the form of oxytetracycline residues was present. Apparently, transfer of plasmids containing tetracycline resistance determinants occurred spontaneously and with subsequent selection pressure a resistant population may develop and manifest itself. It is also a possibility that transfer will increase when small amounts of oxytetracycline are present (29).

It should be stressed that even if there is a possibility of these transfers to take place in nature as indicated, there remains much work to be able to perform a genuine risk assessment, which is rendered difficult when dealing with processes occurring widespread in nature.

ACKNOWLEDGEMENTS

We would like to thank Farah S. Bahrani for excellent technical work. The staff at the fish farms for good collaboration. Søren Sørensen for donating the *E. coli* and *P. putida* strains. Yvonne Agerskov for screening *F. psychrophilum* isolates for Tet determinants K, L, M, O and S. This project was funded in part by The Ministry of Food, Agriculture and Fisheries.

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TABLE 1. Transfer in vitro – filter mating with motile *Aeromonas* spp. as donor and *E. coli* CSH26Rf as recipient

Putative donor					Transfer frequency[a]	Transconjugant	
Isolate	Plasmids		MIC_{OTC}	Tet-determinant		Plasmids	Tet-determinant
	Number	Size of large plasmids, kb					
1-75	2	160	32	A	3.4×10^{-5}, 1.0×10^{-6}	160	A
1-78	5	150	32	E	NT, NT		
1-163	3	30, 100, 150	64	A	NT, NT		
1-231	1	150	512	D	3.8×10^{-7}, 4.4×10^{-8}	150	D
1-250	3	150	64	?	4.9×10^{-8}, 9.0×10^{-7}	150	?
1-318	6	40	64	E	NT, NT		
1-337	2	150	32	A	1.3×10^{-7}, 3.2×10^{-6}	150	A
1-358	2	150	32	A	5.6×10^{-7}, 3.2×10^{-7}	150	A
1-372	6	30,60	32	?	NT, NT		
2-41	1	150	16	?	3.2×10^{-7}, 7.2×10^{-7}	150	?
2-62	8	160	32	A	1.1×10^{-5}, 2.6×10^{-6}	160	A
2-85	2	150	16	A	6.8×10^{-5}, 3.3×10^{-6}	150	A
2-155	2	150	64	?	NT, NT		
2-189	2	150	64	?	1.4×10^{-9}, 4.1×10^{-8}	150	?
2-197	4	50,150	64	?	NT, NT		
2-219	6	150	64	?	NT, NT		
2-280	5	45,150	16	A	6.9×10^{-8}, 1.4×10^{-8}	150	A
2-282	3	150	32	?	NT, NT		
2-321	5	160	32	?	NT, NT		
2-333	3	150	32	?	NT, NT		
2-410	1	140	64	A	NT, NT		
3-30	3	150	16	?	NT, NT		
3-130	6	100	16	?	NT, NT		
3-135	3	150	64	?	NT, NT		
3-217	8	150	32	?	NT, NT		
3-247	10	100	32	?	NT, NT		
3-372	2	150	32	?	1.0×10^{-7}, 6.9×10^{-6}	150	?
4-80	1	150	64	E	NT, NT		
4-97	5	40,55,150	16	E	NT, NT		
4-206	3	150	64	AE	NT, NT		
4-221	1	150	64	A	1.4×10^{-6}, 1.3×10^{-8}	150	A
4-229	4	120	64	A	NT, 7.0×10^{-10}	3, 5, 10, 120	A
4-241	2	40, 150	32	?	NT, NT		
4-280	2	150	32	?	1.3×10^{-7}, 9.4×10^{-6}	150	?
4-302	7	30, 150	32	?	NT, NT		
4-307	1	150	32	A	4.0×10^{-6}, 2.9×10^{-6}	150	A
4-348	5	150	32	?	NT, NT		
4-353	2	60	32	?	NT, NT		
4-354	2	45	32	E	NT, NT		
4-440	1	150	32	A	6.8×10^{-6}, 1.1×10^{-6}	150	A

NT = No transfer detected, a = number of transconjugants per recipient

? = Unidentified determinant

TABLE 2. Transfer in vitro – filter mating with motile *Aeromonas* spp. as donor and other recipients than *E. coli* CSH26Rf

Putative donor	Recipient Isolate	Native plasmids	Transfer frequency
	P. putida		
1-75	KT2442	None	NT, NT
1-163	KT2442	None	NT, NT
	F. psychrophilum		
1-75	2-51	2 small	NT, NT
1-75	3-201	1 small	NT, NT
1-75	4-98	1 small	NT, NT
2-62	2-51	2 small	NT, NT
2-62	3-201	1 small	NT, NT
2-62	4-98	1 small	NT, NT
	Y. ruckeri		
1-75	1-41	2 large + 1 small	NT, NT
1-75	3-48	2 large + 1 small	NT, NT
1-75	4-49	2 large + 1 small	NT, 2.1×10^{-5}
2-62	1-41	2 large + 1 small	NT, NT
2-62	3-48	2 large + 1 small	NT, NT
2-62	4-49	2 large + 1 small	NT, 1.7×10^{-7}
	Motile *Aeromonas* spp.		
1-75	1-58	2 small	NT, NT
1-75	1-181	1 small	1.6×10^{-6}, 3.3×10^{-5}
1-75	4-251	None	NT, NT
1-75	4-251Rf	None	$1. \times 10^{-6}$, 2.8×10^{-6}
2-62	1-58	2 small	NT, NT
2-62	1-126	None	6.8×10^{-5}
2-62	1-181	1 small	1.9×10^{-3}
2-62	4-251	None	4.0×10^{-4}, NT

NT = No transfer detected

TABLE 3. Transfer in simple microcosms incubated at 20°C for 20 hours.

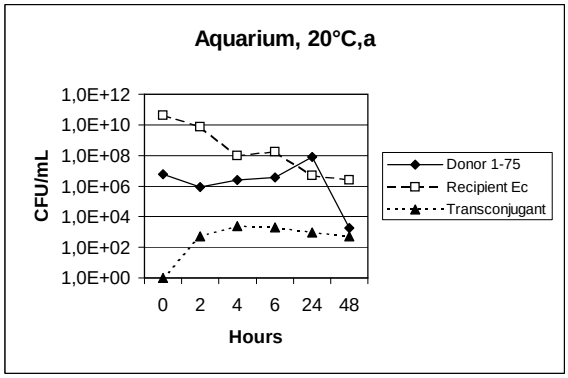
Putative donor	Recipient	Transfer frequency
1-75	*E .coli*	6.0×10^{-8}
	CSH26Rf	2.6×10^{-7}
2-62	*E .coli*	2.5×10^{-9}
	CSH26Rf	2.3×10^{-8}
4-221	*E. coli*	5.2×10^{-8}
	CSH26Rf	NT

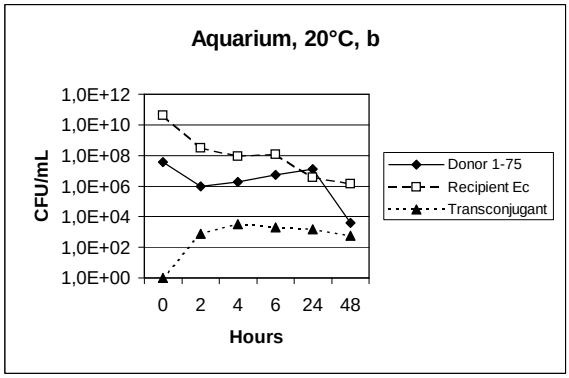
NT = No transfer detected

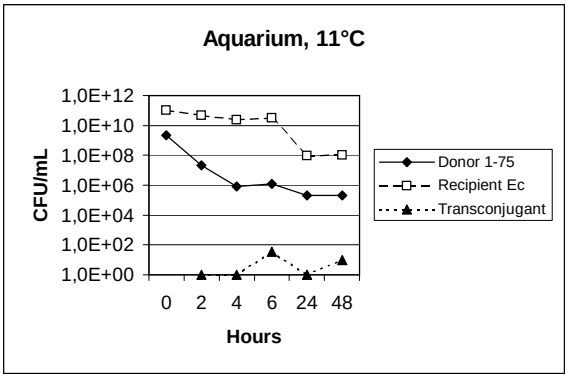
TABLE 4. Transfer in membrane filter chambers

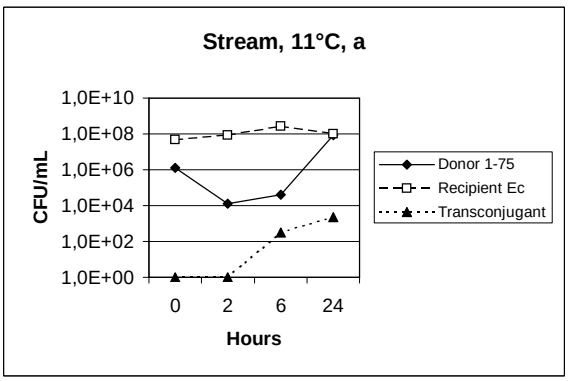
Putative donor	Recipient	Experimental setting		Transfer frequency[a]				
		Set-up	Temp.	2 hours	4 hours	6 hours	24 hours	48 hours
Motile *Aeromonas* 1-75	*E. coli* CSH26Rf	Aquarium	20°C	6.8×10^{-8}	2.4×10^{-5}	1.2×10^{-5}	1.9×10^{-4}	2.1×10^{-4}
				2.7×10^{-6}	3.7×10^{-5}	1.7×10^{-5}	4.1×10^{-4}	4.3×10^{-4}
			11°C	2.2×10^{-11}	0	1.1×10^{-9}	0	1.0×10^{-7}
		Stream	11°C	1.1×10^{-7}	-	1.1×10^{-6}	2.3×10^{-5}	-
				2.4×10^{-6}		1.3×10^{-5}	3.5×10^{-5}	
Motile *Aeromonas* 1-75	*Y. ruckeri* 4-49	Aquarium	20°C	NT, NT	NT, NT	NT, NT	NT, NT	NT, NT
			11°C	NT, NT	NT, NT	NT, NT	NT, NT	NT
Motile *Aeromonas* 1-75	Motile *Aeromonas* 4-251	Aquarium	20°C	6.1×10^{-6}	9.9×10^{-7}	3.8×10^{-4}	0	-
				0	1.3×10^{-4}	5.1×10^{-4}	0	-
			11°C	NT, NT	NT, NT	NT, NT	NT, NT	NT, NT
		Stream	11°C	5.7×10^{-6}	-	1.9×10^{-6}	5.5×10^{-4}	-
				NT		NT	NT	
	Motile ***Aeromonas 4-251Rf***	Stream	11°C	NT, NT	-	NT, NT	NT, NT	-
Motile *Aeromonas* 2-62	*E. coli* ***CSH26Rf***	Aquarium	11°C	NT	NT	-	1.0×10^{-5}	
		Stream	11°C	NT, NT	-	5.9×10^{-8},	9.0×10^{-7}, NT	-
						NT		
Motile *Aeromonas* 2-62	*Y. ruckeri* 4-49	Aquarium	20°C	NT, NT	NT, NT	NT, NT	NT, NT	-
A. salmonicida 950704-2/1	*E .coli* CSH26Rf	Aquarium	11°C	NT	NT	NT	NT	-
		Stream	11°C	NT, NT	-	NT	NT	-
						1.3×10^{-7}		
	Motile *Aeromonas* 4-251Rf	Aquarium						
		Stream	11°C	NT, NT	-	NT, NT	NT	-
A. salmonicida 718	*E. coli* CSH26Rf	Aquarium	20°C	2.8×10^{-8}	4.0×10^{-7}	1.4×10^{-8}	5.0×10^{-9}	-
				3.0×10^{-8}	3.8×10^{-7}	1.0×10^{-8}	7.5×10^{-9}	-
			11°C	5.5×10^{-8}	1.4×10^{-8}	7.0×10^{-9}	5.7×10^{-8}	-
	Motile *Aeromonas* 4-251	Aquarium	20°C	NT, NT	NT, NT	NT, NT	NT, NT	-

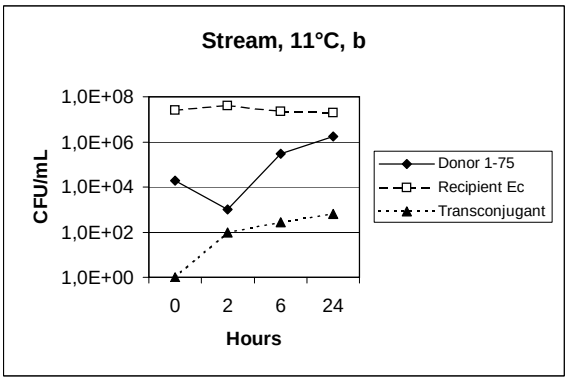
NT = No transfer detected, a = number of transconjugants per recipient, - = not sampled

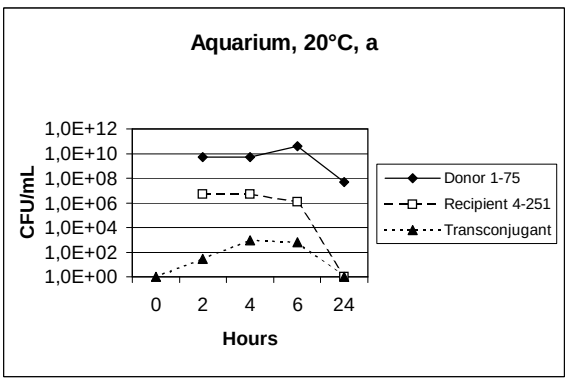


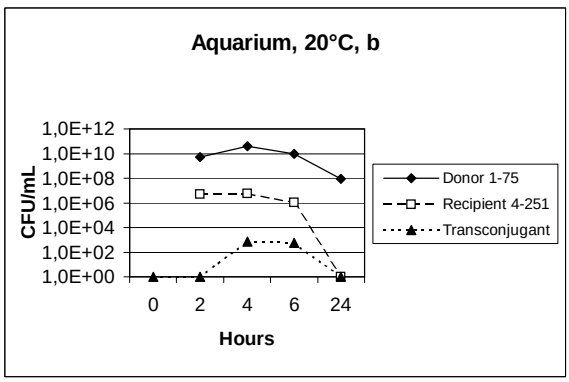


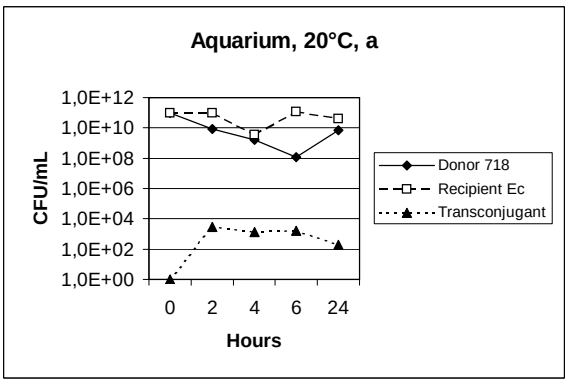












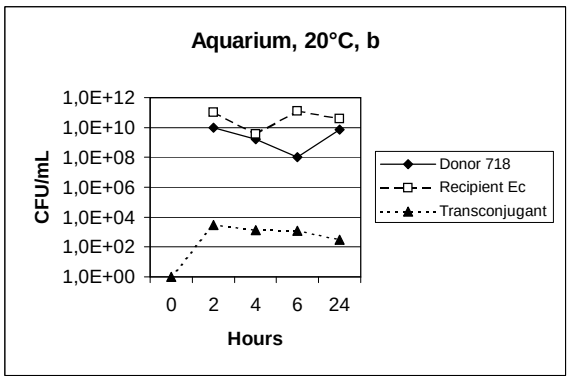


Fig. 1 Number of CFU per ml in various transfer experiments with membrane filter chambers.
a,b = duplicate experiments, Ec = *E. coli*

Efficiency of oxytetracycline treatment in Rainbow Trout experimentally infected with *Flavobacterium psychrophilum* strains having different in vitro antibiotic susceptibilities

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ABSTRACT

The medication effect of oxytetracycline on groups of rainbow trout fry experimentally infected with three strains of *Flavobacterium psychrophilum* was investigated. The infection model was based on intraperitoneal injection of the pathogen and treatment was done using medicated feed resulting in 100 mg oxytetracycline/kg fish for ten days. The three *F. psychrophilum* strains had different antimicrobial susceptibilities and successful treatment was only obtained in the trial using a strain with a MIC_{OTC} of 0.25 μg/ml. No effect of treatment was seen in the group infected with a strain having MIC_{OTC} of 8.0 μg/ml and only little effect was seen when the strain MIC_{OTC} was 4.0 μg/ml. This shows that it is valid to predict treatment efficiency of OTC from in vitro data facing an outbreak of rainbow trout fry syndrome. The importance of doing susceptibility testing is emphasized, and as shown the selection of media for antimicrobial susceptibility testing of *F. psychrophilum* is important.

Keywords: Flavobacterium psychrophilum; Bacterial cold water disease; Rainbow trout fry syndrome; Experimental infection; Oxytetracycline; Minimum inhibition concentration; Rainbow trout

1. INTRODUCTION

Flavobacterium psychrophilum (Bernardet et al., 1996) is the causative/etiological agent of Bacterial Cold Water Disease (BCWD) seen primarily in salmonids. BCWD was first described by Davis in 1946 in USA (Borg, 1948), and is now enzootic in several countries (Holt et al., 1993). In Europe the disease in fry is called rainbow trout fry syndrome (RTFS), and has for 15 years caused serious problems in rainbow trout (*Oncorhynchus mykiss* Walbaum) hatcheries (Bernardet et al., 1988; Lorenzen et al., 1991; Bruno, 1992; Toranzo and Barja, 1993; Wiklund et al., 1994; Sarti and Giorgettii, 1996). In Denmark it has become one of the main causes of losses in rainbow trout fry and fingerlings (Lorenzen, 1994; Dalsgaard and Madsen, 2000). Traditionally, oxytetracycline (OTC) incorporated in fish feed has been the drug of choice for treatment of RTFS (Holt et al., 1993). Progressively the efficacy of oxytetracycline dropped and as described (Bruun et al., 2000) between 60 and 75% of *F. psychrophilum* isolated from Danish fish farms in the years 1994, 1995, 1997 and 1998 was resistant to OTC. Resistance in *F. psychrophilum* to OTC was defined using in vitro determination of the susceptibility and an arbitrarily established breakpoint partly based on pharmacokinetic data from the literature and partly on the distribution of these strains in two separate clusters (Bruun et al., 2000). To demonstrate the usefulness of these in vitro results it seemed reasonable to perform in vivo treatment trials. This investigation is a preliminary study to assess the correlation existing between in vitro susceptibility results for OTC and clinical laboratory data. This work uses a previously introduced experimental infection model (Madsen and Dalsgaard, 1999) to examine the effect of OTC treatment on rainbow trout fry with RTFS caused by three strains of *F. psychrophilum*. No similar studies have apparently been performed on fish infected with *F. psychrophilum*.

2. MATERIALS AND METHODS

2.1. Bacterial strains

Three Danish *Flavobacterium psychrophilum* strains isolated from rainbow trout at the same freshwater farm in 1994 and 1995 were used in the experiment. The strains selected were all virulent according to previous experimental infections (Madsen and Dalsgaard, 2000) and carried different susceptibilities to oxytetracycline (Bruun et al., 2000). In addition differences in serotypes and plasmid profiles were observed (Table 1).

Preparation of bacterial inoculums was done as described by Madsen and Dalsgaard (1999). Strains were inoculated from -80°C into 10 ml tryptone yeast extract salts (TYES) broth (Holt et al., 1993). All incubations were at 15°C with shaking. After incubation for 72 hours, 0.5 ml of the broth culture were inoculated into 100 ml TYES broth and further incubated for 48 hours before adjusting to the absorbance 0.3 at 525 nm. This broth with approximately 10^8 CFU/ml was diluted 1000 fold with TYES and 0.05 ml of this was injected intraperitonally in each fish. Simultaneously bacterial counts were made by the spread plate method to determine the inoculum size (infectious dose) expressed as CFU/fish (Table 1).

2.2. Susceptibility testing

MIC values were obtained using the standardized dilution method previously described (Bruun et

al., 2000). Diffusion tests were performed using diluted Müller-Hinton agar (MDMH) (Difco Laboratories, Detroit, USA; Bruun et al., 2000) and TYES added 1.1% agar, respectively. Commercial discs with a diameter of 9 mm and containing 80 µg OTC (Neo-Sensitabs®, Rosco Diagnostica, Denmark) were used as previously described by Dalsgaard and Madsen (2000).
All tests were done in duplicate and mean values are given as results (Table 1).

2.3. Fish and fish tank facilities

Rainbow trout fry were received at the laboratory with an average weight of one gram from a Danish fish farm without any previous history of RTFS. The stock fish were kept in larger fish tanks, fed restrictively and transferred to the smaller tanks at the beginning of the experiment. After experimental infection, replicate groups of approximately 50 rainbow trout were kept at 12°C in 30 l tanks with separate air supply and recirculating water. Fish of 3 grams ± 0.3 g were used. Experimental set-up and number of fish is shown (Table 2).

2.4. Experimental infection

The fish were anaesthetized with 3-aminobenzoic acid ethyl ester (Sigma A-5040) and subsequently injected i.p. with either 0.05 ml of one of the three strains in standardised whole cell bacterial culture or 0.05 ml sterile TYES as control (Madsen and Dalsgaard, 1999).

2.5 Treatment (Table 2)

Fish were fed 1% of the biomass daily with either plain Ecostart 17 (Biomar A/S) or medicated Ecostart 17 (OTC 1%, Biomar A/S) from day 1 to 10 succeeded by non-medicated Ecostart 17 in the remaining experimental period. Thus the treatment started the day after the experimental infection and the dose equalled 100 mg/kg fish for ten days.
Deaths were recorded daily and dead fish were examined. Necropsy involved gross external and internal examination and microbiological confirmation of RTFS. Tissue from spleen, kidney and brain were sampled aseptically with a sterile loop and inoculated onto TYES agar. The plates were incubated at 15 °C for up to 10 days and pure culture of typical yellow colonies were indicative of RTFS. The experiment was terminated after 42 days where remaining fish were euthanised using an overdose of 3-aminobenzoic acid ethyl ester. Necropsy including bacteriological examination was performed on all fish surviving infection, whereas only five fish from each of the control tanks were examined.

2.6. Statistics
Chi-square tests were used to compare the groups.

3. RESULTS

The results are presented in Fig.1, as cumulative mortality as a function of time. As no deaths occurred the last 14 days the figure x-axis is limited to 28 days. The data for each strain of *F. psychrophilum* are given in separate diagrams and the development in each fish tank can be followed throughout the experimental period. Duplicate tanks yielded similar results ($p > 0.05$, chi-square test) apart from tanks 3 and 4 and the two controls ($p < 0.05$, chi-square test).

Regarding the strain 8/8a with a MIC value of 0.25 µg/ml classified as susceptible to OTC, the figure shows a gradual but high rise in cumulative mortality. All fish (except one) receiving no treatment (tanks 1 and 2) were dead after three weeks. The surviving fish in tank 1 and 2 were negative for *F. psychrophilum*. Fish infected with strain 8/8a and subsequently treated with OTC incorporated into the feed pellets showed no (tank 3) or limited (tank 4) rises in cumulative mortality. One fish from tank 3 died on day 11 and after bacteriological examination only two CFU were detected from the spleen inoculum. These were presumed to be *F. psychrophilum* but were not further characterised. In tank #4 deaths were recorded on day 6 (three fish) and one fish each on days 11, 14, 17, 18 and 19. Bacteriological examination from all these fish yielded pure culture of *F. psychrophilum*-like colonies.

Tanks 5 to 8 contained fish infected with strain 50/9b, considered resistant to OTC with a MIC value of 8.0 µg/ml. High virulence was observed with onset of mortality after 5 days, and most fish deceased after two weeks. Without treatment cumulative mortality of 100% was seen in tank 5 and 6. Fish treated with OTC were not cured and the mortality in tanks 7 and 8 reach the same levels.

The results obtained from tanks 9 to 12 were in between the previous results. The OTC susceptibility of strain 68/4b was in between the susceptibilities of the other strains as well with a MIC of 4.0 µg/ml, still classified as resistant. The virulence was high with onset of mortality on day 5 and cumulative mortality of 96 % in tanks 9 and 10. Medication gave a limited effect and cumulative mortality reached 32 and 38% in tanks 11 and 12 where OTC had been administered.

Problems with mortality in control-tank 14 were observed from day 20 to 24 where a total of 7 fish died. Bacteriological examination yielded no *F. psychrophilum* or other fish pathogens.

Several yellow colonies were isolated from the 170 fish sacrificed at the end of the experiment but only three isolates did comply with the microbiological confirmation of RTFS in combination with *F. psychrophilum* specific PCR (Toyama et al., 1994; Wiklund et al., 1999). The three verified *F. psychrophilum* isolates originated from one fish in tank #4 (spleen and kidney) and one from tank #12 (brain).

Necropsy yielded either no specific findings or petechiae and/or enlarged spleen consistent with RTFS septicaemia.

Discrepancy was observed in the inhibition diameters found using MDMH agar and TYES agar (Table 1). Diameters on MDMH agar were 55, 40 and 48 mm, whereas TYES agar results were 42, 32 and 33 mm, respectively.

4. DISCUSSION

Previous investigations of antimicrobial resistance in *F. psychrophilum* have only dealt with the in vitro aspect. Various methods for determining antimicrobial susceptibility have been used and strains have subsequently been classified as resistant or sensitive (in some cases intermediate). MIC values can be useful indicators of the probable clinical efficacy, but these results have not been verified by in vivo testing (Rangdale, 1995; Schmidtke and Carson, 1995; Lorenzen et al., 1997; Rangdale et al., 1997). Other authors report successful use of oxytetracycline in field outbreaks of RTFS (Holt et al., 1993; Lorenzen et al., 1991; Rangdale, 1994) but no correlation between MIC-values and the in situ efficacy have been done. Finally several authors report on the pharmacokinetics and bioavailability of oxytetracycline in rainbow trout often to determine withdrawal times (Abedini et al., 1998; Jacobsen, 1989; Rogstad et al., 1991, Uno et al., 1992). These data of achievable serum concentrations in combination with in vitro determined MIC-values can be used to estimate/determine treatment efficacy (Bruun et al., 2000). This study shows that this theoretical approach can be verified in vivo, at least in this experimental setting.

The infection model used was shown to be reliable and can be used to study treatment efficiency in fish suffering from RTFS. Obviously using intraperitoneal injection of the pathogen evades several host defence mechanisms, and this experimental infection cannot be used to study the initial steps of the pathogenesis. Strains belonging to serotypes Fd or Th are equally virulent according to previous experimental infection experiments (Madsen and Dalsgaard 1999, 2000)

The breakpoint of 0.25 µg/ml previously recommended (Bruun et al., 2000) is probably rather low as some effect was seen when treating fish infected with strain 68/4b having MIC of 4 µg/ml. The present study was done in laboratory aquaria and the treatment efficacy is most likely lower in vivo due to various factors, e.g. due to larger feed spill and subsequent degradation and/or adsorption of the OTC. If a diffusion method with tablets containing antimicrobial agents is to be used it is important to use diluted Müller-Hinton agar and not TYES agar as inhibition zones are unreliable on TYES agar (Table 1, Dalsgaard 2001). Using the agar diffusion method applying Neo-Sensitabs® (Rosco Diagnostica) with 80 µg OTC/disc the following breakpoint diameters were recommended: susceptible>47 mm and resistant<42 mm (Dalsgaard and Madsen 2000). This also shows that using TYES agar for susceptibility testing would classify all three isolates as resistant.

It should be noted that OTC only are used rarely for therapy of RTFS in Danish fish farms for the later years as resistance has become widespread.

The fish farm from which 50/9b were isolated had a history of recurrent RTFS outbreaks in the previous months. Each time OTC treatment was instigated and mortality dropped but eventually new outbreaks occurred. Eventually the fish were treated with amoxicillin, and no more outbreaks were observed that year. 50/9b were isolated from an apparently healthy fish a month later, and the detected OTC resistance could be a result of this history. The large plasmid carried by the resistant strain 50/9b could be a resistance plasmid but no curing or transfer experiments were performed to confirm this.

Survival of initially OTC susceptible *F. psychrophilum* in fish treated with OTC could happen if:

1. The bacteria acquire resistance towards OTC during the initial treatment period. As OTC resistance determinants often are located on mobile elements (transposons or plasmids) in various bacterial species, it is possible that gene transfer has occurred e.g. from the indigenous bacterial flora in the fish intestine, gills or mucus layer. Unfortunately, bacterial isolates from deceased fish in tanks 3, 4, 11 and 12 were not examined further for existence of resistance plasmids. The authors have rarely detected

large plasmids in *F. psychrophilum* strains and are unaware of any previous descriptions of R-plasmids in this species. Thus it is speculated that resistance to oxytetracycline in *F. psychrophilum* is caused by uptake of transposons carrying tetracycline resistance determinants or the resistance is caused by other unspecific changes in membrane permeability.

2. The bacteria reside in the brain where OTC concentrations are significantly lower than in the blood due to the blood-brain barrier. Apparently no work has been done in fish, but it is well known that concentrations of pharmaceuticals vary from one organ to the next. The OTC concentration in brain tissue is probably low and *F. psychrophilum* was often isolated from the brain in this study as well as in clinical specimens from RTFS outbreaks in the field (Dalsgaard and Madsen, 2000).
3. The bacteria survive in the surrounding water or in the biofilm on fish tank surfaces, where OTC concentrations most likely are considerable lower than in the fish serum. Samples were not taken from the fish tank water or biofilm, but *F. psychrophilum* is previously isolated from natural aquatic environments (Bruun et al., 2000), and the polymer matrix of a biofilm has the ability to protect the embedded bacteria against antimicrobial agents and other exogenous substances (Marshall, 1997).

Antibiotic treatment should be limited for several reasons. Two of the things to be aware of is to limit development and spread of antimicrobial resistance in naturally occurring (indigenous) and fish pathogenic bacteria and to avoid residues in fish for human consumption. Preventive measures are preferable in the form of good stock management and eventually vaccination, and will keep disease outbreaks to a minimum. If bacterial disease emerges treatment with antibiotics is necessary.

5. CONCLUSION

The results of this study contributes to the understanding of antimicrobial therapy in rainbow trout infected with *F. psychrophilum*, which was previously based on experience and empirical use of data from other bacterial and animal species. It is shown that it is valid to predict whether or not OTC will be efficient in treating RTFS from in vitro data of susceptibility of the strain responsible for the outbreak in question. This will most likely be the case with other antimicrobial agents, fish species and bacterial pathogens but it has yet to be assessed. The importance of doing susceptibility testing when facing an outbreak at a fish farm is obvious, but as shown the selection of media for antimicrobial susceptibility testing of *F. psychrophilum* is important.

ACKNOWLEDGEMENTS

The authors would like to thank Farah S. Bahrani, Kirsten Kaas and Ann Charlot Ejsing for excellent technical work. Helma Slierendrecht at Biomar for providing the medicated feed. This project was funded in part by The Ministry of Food, Agriculture and Fisheries.

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Table 1

F. psychrophilum strains used in the experiment

Strain	8/8a	50/9b	68/4b
MIC_{OTC} (µg/ml)	0.25	8	4
MDMH agar	55[A]	40[A]	48[A]
TYES agar	42[A]	32[A]	33[A]
Inoculums (CFU/fish)	$1.3x10^4$	$4.0x10^4$	$2.0x10^4$
Virulent [B]	+	+	+
Serotype [B]	Fd	Th	Th
Plasmid	3.3	3.3 + 50	3.3
Origin	Diseased fish March 1994 Kidney	Apparently healthy fish November 1994 Brain	Diseased fish March 1995 Brain

[A] Inhibition zones in mm with 80 µg OTC discs

[B] See Madsen and Dalsgaard, 2000

Table 2

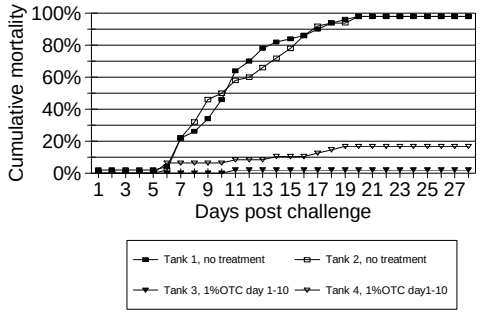
Experimental set-up

Tank	Status	# of fish		Feed
		Start	End	
1, 2	8/8a	50, 50	1,1	Ecostart 17
3, 4	8/8a	50, 48	49,40	OTC (1%)
5, 6	50/9b	50, 50	0,0	Ecostart 17
7, 8	50/9b	51, 48	0,0	OTC (1%)
9, 10	68/4b	51, 47	2,2	Ecostart 17
11, 12	68/4b	50, 50	34,31	OTC (1%)
13	control	50	49	Ecostart 17
14	control	50	42	OTC (1%)

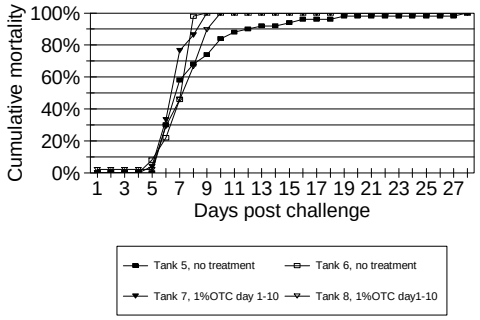
OTC (1%) = Ecostart 17 + 1 % OTC

Fig. 1
Experimental infections: cumulative mortality as a function of time. The data for each strain of *F. psychrophilum* are shown in separate diagrams.

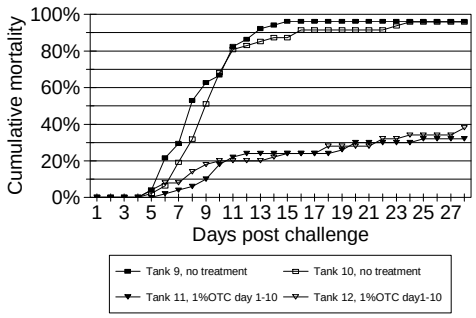
8/8a
OTC susceptible, MIC = 0,25µg/ml



50/9b
OTC resistant, MIC = 8 µg/ml



68/4b
OTC resistant, MIC = 4 µg/ml



Control