Occurrence and fate of antibiotic resistant bacteria in sewage

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Preface

This report contains the results of a project conducted at the Department of Veterinary Microbiology of The Royal Veterinary and Agricultural University (RVAU). The project is composed of three parts (I, II and III). After an introductory chapter on antibiotics, antibiotic resistance and project structure (chapter 1), the methodology used is described in chapter 2. The outcomes of each part of the project are then reported and discussed separately in chapters 3, 4 and 5.

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The results of the project have been published in national and international journals and presented at scientific meetings as listed in Annex 1.

Summary and conclusions

In this project, we investigated different aspects concerning the occurrence and fate of antibiotic resistant bacteria in sewage:

- the effects of waste effluent from a hospital and a pharmaceutical plant on the prevalence of resistant *Acinetobacter* in the recipient sewers (Part I)

- the effects of tertiary sewage treatment on total numbers and percentages of resistant bacteria (Part II)

- the survival in natural aquatic habitats of multiple-resistant bacteria originating from municipal sewage effluents, and more in general the impact of such effluents on the spread of antibiotic resistance (Part III).

Part I

In Part I of the project, *Acinetobacter* was used as a bacterial indicator for monitoring antibiotic resistance in the sewers receiving waste effluent from two potential sources of resistant bacteria and/or antibiotic residues: a hospital and a pharmaceutical plant manufacturing antibiotics. The choice of *Acinetobacter* was prompted by the normal occurrence of these organisms in water and their remarkable ability to develop antibiotic resistance.

The levels of susceptibility to six antibiotics were determined by the disc-diffusion method in 385 *Acinetobacter* isolates from sewage collected upstream and downstream from the discharge points of the hospital (n=180) and the pharmaceutical plant (n=205). Isolates from the sewers at the pharmaceutical plant were further analysed by plasmid DNA profiling and phenotypic tests to detect any changes in the distribution of *Acinetobacter* species/strains associated with the discharge of waste effluent from this source.

Statistical analysis of the data from antibiotic susceptibility testing showed that the discharge of waste effluent from the pharmaceutical plant was associated with a statistically significant increase in the prevalence of both single and multiple antibiotic resistance profiles amongst *Acinetobacter* isolates from the recipient sewers (logistic regression P < 0.01). This increase of antibiotic resistance was observed throughout the period of study and was evident up to 250 m downstream from the discharge point. Strains isolated downstream from the pharmaceutical plant discharge point also demonstrated different plasmid profiles and phenotypic traits compared with those isolated upstream.

In contrast, the hospital waste effluent increased only the prevalence of resistance to one antibiotic, namely oxytetracycline, amongst *Acinetobacter* isolates from the recipient sewers. Furthermore, the increase in tetracycline resistance observed immediately downstream from the hospital discharge point was significantly reduced 500 m further downstream from the discharge point.

Based on this evidence, it was concluded that the discharge of waste effluent from the pharmaceutical plant caused an increase in the prevalence of both single and multiple antibiotic resistance amongst *Acinetobacter* spp. in the recipient sewers and had a higher impact on antibiotic resistance compared with the discharge of hospital waste effluent. Furthermore, waste effluent from the pharmaceutical plant determined a change in the distribution of *Acinetobacter* species/strains in the sewers, probably due to introduction of either antibiotic residues or resistant strains.

Part II

Part II of the project dealt with the effects of tertiary sewage treatment on the prevalence of resistant bacteria in two large-scale municipal treatment plants during a period of six months. Total and relative numbers of resistant bacteria in raw sewage, treated sewage and anaerobically digested sludge were determined by bacteriological counts on agar media with and without inclusion of ampicillin, tetracycline, gentamicin or all three antibiotics. Two different agar media, one selective for coliforms (MacConkey agar) and one selective for *Acinetobacter* (Baumann agar), were used in order to study the effect of treatment on different bacterial populations. In addition, the levels of susceptibility to 14 antibiotics were determined by the disc-diffusion method in 442 *Acinetobacter* isolates identified by colony hybridisation with a genus-specific DNA probe.

Depending on the different antibiotics and media used for bacteriological counts, the total numbers of resistant bacteria ranged between 10 to 1000-fold lower in treated sewage than in raw sewage. For both bacterial populations under study, the prevalence of resistant bacteria in treated sewage and digested sludge were not significantly higher than in raw sewage. On the contrary, the prevalence of ampicillin-resistant acinetobacters (i.e. presumptive *Acinetobacter* not identified at the genus level) was significantly reduced by sewage treatment at one plant (linear regression $P < 0.05$). Similarly, sludge treatment determined a reduction in the prevalence of ampicillin-resistant acinetobacters, as well as ampicillin- and gentamicin-resistant putative coliforms (linear regression $P < 0.05$).

The results obtained by bacteriological counts were confirmed by antibiotic susceptibility testing of *Acinetobacter* isolates. Based on logistic regression analysis, the frequencies of antibiotic resistance in isolates from treated sewage and digested sludge were not significantly higher in comparison with those in isolates from raw sewage. Comparison of the levels of resistance to 14 antimicrobial agents between isolates obtained from raw and treated sewage allowed detection of a statistically significant increase in the prevalence of antibiotic resistance to only one antibiotic (nalidixic acid) and restricted to one plant. It can therefore be concluded that tertiary sewage treatment did not determine a selection for resistant bacteria. In accordance, sewage treatment appears to reduce numbers of bacteria irrespective of their susceptibility to antibiotics.

Although the results of the study clearly indicated that the overall prevalence of antimicrobial resistant bacteria was not increased by sewage treatment, the final effluent of one plant was found to contain low numbers (10 to 10^2 CFU/ml) of bacteria resistant to ampicillin, gentamicin and tetracycline, collectively. This multiple resistance phenotype is not likely to occur naturally in aquatic bacteria, as suggested by the absence of bacterial growth following inoculation of freshwater and seawater samples on agar plates containing these

three antibiotics. Consequently, it was decided to investigate the ability of such multiple-resistant bacteria to survive in natural aquatic environments (Part III).

Part III

In the final part of the project, three multiple-resistant strains isolated from treated sewage were investigated for their ability to survive in natural waters and retain antibiotic resistance. In parallel, survival experiments in laboratory seawater microcosms and membrane-filter chambers immersed in a freshwater pond were also carried out. The three strains were representative of three different bacterial species: *Acinetobacter johnsonii*, *Escherichia coli* and *Citrobacter freundii*. The multiple resistance patterns of these strains were used as selective markers for their detection among the indigenous bacteria. The experiments were performed using low bacterial inoculums (10^3 to 10^4 CFU ml^{-1}) appropriate to reproduce the actual conditions occurring when treated sewage is discharged into natural aquatic recipients.

Two of the three multiple-resistant strains (*Escherichia coli* and *Citrobacter freundii*) survived for at least one month in the seawater microcosms and the freshwater pond, whereas, the *Acinetobacter johnsonii* strain survived for shorter times in both settings. The results demonstrated that some multiple-resistant strains occurring in municipal sewage effluents were able to survive for relatively long periods following their release into natural aquatic habitats. The strains survived longer in autoclaved water better than in untreated water, suggesting that the presence of the indigenous microflora affected survival, presumably due to antagonism or predation. However, the strains maintained their multiple resistance properties following one month of incubation under natural conditions, indicating that stress and nutrient depletion did not affect the stability of their resistance phenotypes.

An aspect of the study in Part III focused on the possibility that antibiotic resistance genes occurring in sewage could be transferred to bacteria living in natural aquatic environments. This was studied by laboratory mating experiments. Ten unrelated tetracycline-resistant *Acinetobacter* strains isolated from sewage (n=10) were mated with a tetracycline-sensitive *Acinetobacter* strain isolated from an unpolluted stream. Only two out of ten donor strains were able to transfer tetracycline resistance under laboratory conditions (in vitro). In one instance, transfer of tetracycline resistance was associated with relocation of multiple small plasmids from the donor to the recipient strain, whereas, in another, transfer was apparently not mediated by plasmid conjugation. These results confirmed that sewage is a possible vehicle for the dissemination of antibiotic resistance genes in the indigenous microflora of aquatic habitats. However, the limited number of strains used in the mating experiments did not allow conclusions to be drawn on the occurrence of this phenomenon in nature.

The impact of municipal sewage effluents on the spread of antibiotic resistance was further investigated by comparing the occurrence of resistant bacteria in blue mussels exposed to sewage effluents and in blue mussels originating from unpolluted sites. Blue mussels were selected as a biological niche due to their ability to harbour high numbers of bacteria through daily filtration of large volumes of water. Higher percentages of ampicillin-resistant bacteria were found in mussels exposed to treated sewage (12.9 to 95.5%) in comparison with mussels not exposed to treated sewage (1.5 to 5.4%), whereas, the percentages of gentamicin- and tetracycline-resistant bacteria

were low (<3%) independent of the origin of the mussels. Small traces (#0.1%) of multiple-resistant bacteria were found only in mussels exposed to treated sewage, suggesting that municipal sewage effluents are potentially a source for the spread of these bacteria in the aquatic environment. The isolation of resistant bacteria was generally higher in mussels collected from the immediate proximity of the outlets of sewage effluents compared with mussels collected at 100 m from the outlets, indicating a correlation between prevalence of resistant bacteria and distance from the outlet.

Conclusions

The results of this project indicate that the occurrence of single and multiple-resistant bacteria in sewage can be increased by the discharge of waste effluent from pharmaceutical plants producing or manufacturing antibiotics. To our knowledge, this was the first study demonstrating an impact of antibiotic manufacturing on the occurrence of resistant bacteria in sewage. The observed increase in the prevalence of resistant *Acinetobacter* could have been due to the presence in the effluent of resistant bacteria selected inside the plant by the presence of high antibiotic concentrations. Alternatively, antibiotic residues may have caused a selection of resistant bacteria in the recipient sewers, or a combination of both. These results indicate that waste effluents from pharmaceutical plants manufacturing antibiotics are an important source for the occurrence and selection of resistant bacteria in sewage. As this study was conducted on a single pharmaceutical plant, further investigation is needed to assess the role of antibiotic manufacturing on selection and/or introduction of resistant bacteria in sewage.

The occurrence and fate of resistant bacteria in sewage should be given carefully consideration due to the ubiquitous nature of bacteria. Sewage is a convenient and suitable vehicle for the dissemination of resistant bacteria, in that it connects antibiotic selective environments, such as hospitals, chemical industries, farms and slaughterhouses to natural environments. Risk assessment was not included as an objective in this study. However, risks for human health may result from the dissemination in the environment of resistant bacteria occurring in sewage and the possible contamination of bathing and drinking water with these organisms.

Our investigation indicates that sewage treatment causes a reduction in the total numbers of resistant bacteria without increasing their percentage in treated sewage compared with raw sewage. Therefore, it appears that treatment of sewage has a positive effect in limiting the dissemination of resistant bacteria.

However, the investigation also demonstrates that:

- Multiple-resistant bacteria occurring in raw sewage can survive treatment and reach natural aquatic environments by municipal sewage effluents.

- Multiple-resistant bacteria occurring in municipal sewage effluents can survive for relatively long periods and maintain their resistance properties following introduction into natural aquatic habitats.

- Bacteria resistant to three or four different classes of antibiotics were found in shellfish exposed to municipal sewage effluents but appear to

be absent in shellfish and water originating from unpolluted aquatic environments.

- Resistant bacteria originating from sewage are able to transfer genes encoding antibiotic resistance to susceptible bacteria living in unpolluted aquatic habitats.

The results from our investigations underline the need to assess the impact of municipal sewage effluents on dissemination of multiple-resistant bacteria. Future studies monitoring the environmental impact of municipal sewage effluents on the spread of antibiotic resistance should focus attention on multiple-resistant bacteria rather than bacteria resistant to single antibiotics. Furthermore, multiple-resistant strains occurring in municipal sewage effluents should be investigated for their ability to transfer resistance genes to aquatic microbial communities under *in vivo* conditions. Similarly, the fate of resistant bacteria and resistance genes occurring in sewage sludge intended for agricultural use should be studied following their introduction into natural soil habitats. Finally, the relative importance and contribution of resistant bacteria in the aquatic environment and the consequent risk for resistance problems in veterinary and human medicine should be assessed.

Sammendrag og konklusioner

I dette projekt har vi undersøgt forskellige aspekter vedrørende forekomst og overlevelse af antibiotika-resistente bakterier i spildevand, herunder:

- hvilken effekt udledning af spildevand fra et hospital og en farmaceutisk virksomhed havde på prævalensen af antibiotika-resistente *Acinetobacter* i kloaksystemet (Del I)

- effekten af tertiær spildevandsbehandling på det totale og procentvise antal resistente bakterier (Del II)

- overlevelsen i naturlige akvatiske miljøer af multi-resistente bakterier fra byspildevand og indflydelsen af byspildevand på spredningen af antibiotika-resistente bakterier (Del III).

Del I
I Del I af projektet blev *Acinetobacter* anvendt som bakteriel indikator til overvågning af antibiotikaresistens i kloakker. Kloakkerne modtog spildevand fra et hospital og en farmaceutisk virksomhed, som forarbejdede antibiotika-holdige produkter. Begge er således potentielle kilder til resistente bakterier og/eller antibiotika-holdigt spildevand:. Baggrunden for valget af *Acinetobacter* som indikator var bakterieslægtens normale forekomst i vandmiljøer og dens udtalte evne til at udvikle antibiotikaresistens.

Resistens overfor seks antibiotika blev bestemt ved tabletdiffusionsmetode af 385 *Acinetobacter* bakterier isoleret opstrøms og nedstrøms for udledning af spildevand fra hospitalet (n=180) og den farmaceutiske virksomhed (n=205). Bakterieisolater i spildevandet fra kloakken ved den farmaceutiske virksomhed blev endvidere karakteriseret ved plasmid analyse og fænotypiske tests til påvisning af ændringer i fordelingen af *Acinetobacter* populationer som følge af udledning af spildevand fra virksomheden.

Statistisk analyse af resultaterne fra antibiotikaresistens undersøgelserne viste, at udledning af spildevand fra den farmaceutiske virksomhed medførte en stigning i prævalensen af såvel enkelt som multi-resistente *Acinetobacter* i kloakspildevandet nedstrøms for virksomheden (logistisk regression P < 0,01). Denne stigning i antibiotikaresistens blev observeret gennem hele undersøgelsesperioden og persisterede 250 m nedstrøms for stedet for virksomhedens spildevandsudledning.

Dette var i modsætning til udledning af hospitalsspildevand, som kun medførte en stigning i prævalensen af oxytetracyklin-resistente *Acinetobacter* i kloakspildevandet. Den observerede stigning i tetracyklinresistens umiddelbart nedstrøms for spildevandsudledningen blev dog signifikant reduceret 500 m nedstrøms for udledningsstedet. Bakterier isoleret nedstrøms for den farmaceutiske virksomhed havde forskellige plasmidprofiler og fænotypiske egenskaber sammenlignet med *Acinetobacter* bakterier isoleret opstrøms.

Det kunne således konkluderes på baggrund af undersøgelsens resultater, at udledningen af spildevand fra den farmaceutiske virksomhed medførte en

stigning i forekomsten af antibiotika-resistente bakterier på grund af tilførsel af antibiotika-holdigt spildevand og/eller tilførsel af antibiotika-resistente bakterier. Endvidere medførte spildevandsudledningen også ændringer i forekomsten og fordelingen af forskellige *Acinetobacter* fænotyper.

Del II

I projektets Del II undersøgte vi effekten af tertiær spildevandsbehandling på prævalensen af antibiotika-resistente bakterier i to store spildevandsrensningsanlæg gennem en 6 måneders periode. Det totale og relative antal resistente bakterier i ubehandlet spildevand, behandlet spildevand og anaerob-behandlet slam blev bestemt ved kimtællinger på agarmedier med og uden følgende antibiotika: ampicillin, tetracyklin, gentamicin, samt agarmedier med alle tre antibiotika inkluderet samtidigt. MacConkey agar blev anvendt til påvisning af koliforme bakterier og Baumann agar til påvisning af *Acinetobacter*. Anvendelse af de to agarmedier muliggjorde undersøgelse af effekten af spildevand-og slambehandling på forskellige bakteriepopulationer. Desforuden, blev følsomheden hos 442 *Acinetobacter* isolater overfor 14 antibiotika bestemt ved tabletdiffusionsmetode. Identiteten af de testede *Acinetobacter* isolater var forinden blevet fastlagt ved kolonihybridisering med en genus-specifik DNA probe.

Det totale antal resistente bakterier var mellem 10 og 1.000 gange lavere i behandlet end i ubehandlet spildevand afhængig af de anvendte antibiotika og påviste bakteriepopulationer. Prævalensen af resistente koliforme bakterier og *Acinetobacter* i behandlet spildevand og behandlet slam var ikke signifikant højere end i ubehandlet spildevand. Faktisk blev prævalensen af ampicillin-resistente acinetobacter (suspekte *Acinetobacter,* som ikke er identificeret på slægts niveau) signifikant reduceret ved spildevandsbehandling i et af de to undersøgte anlæg (lineær regression P<0,05). Slambehandling medførte også en reduktion i prævalensen af ampicillin-resistente acinetobacter, samt en reduktion i ampicillin- og gentamicin-resistente suspekte koliforme bakterier (lineær regression P<0,05).

Resultaterne fra kimtællinger på agarmedier med antibiotika blev underbygget af resistensbestemmelserne af *Acinetobacter* isolater ved tabletdiffusion. Logistisk regressionsanalyse viste, at frekvensen af antibiotikaresistens i bakterieisolater fra behandlet spildevand og behandlet slam ikke var signifikant højere sammenlignet med isolater fra ubehandlet spildevand. Ved sammenligning af resistensprofilerne hos isolater fra ubehandlet og behandlet spildevand overfor de 14 antimikrobielle stoffer, fandtes der kun for et enkelt antimikrobielt stof, nalidixcan syre, i et af rensningsanlæggene, en signifikant højere prævalens af resistente bakterier i behandlet spildevand sammenlignet med ubehandlet spildevand. Det kan således konkluderes, at tertiær spildevandsrensning ikke medfører en selektion for resistente bakterier. Med andre ord ser spildevandsrensning ud til at reducere bakterieantallet uafhængig af deres følsomhed overfor antibiotika.

Selvom resultaterne klart viser, at den overordnede prævalens af resistente bakterier ikke steg som følge af spildevandsrensning, så indeholdt det udledte behandlede spildevand lave bakterieantal ($10-10^2$ CFU/ml), som var resistente overfor ampicillin, gentamicin og tetracyklin. Sandsynligheden for at sådanne multi-resistente bakterier forekommer i naturen er meget lille. Dette blev understreget af, at der ikke blev set bakterievækst efter udsæd af ferskvands- og saltvandsprøver på agarmedier med de tre antibiotika. På baggrund af

fundet af disse multi-resistente bakterier, besluttede vi, at undersøge deres overlevelsesevne i naturlige akvatiske miljøer (Del III).

Del III

I projektets Del III, blev tre multi-resistente bakteriestammer undersøgt for deres overlevelse og evne til at bibeholde antibiotikaresistens i saltvand i laboratorieforsøg og i membranfilterkamre nedsænket i en ferskvandsdam. De valgte bakteriestammer repræsenterede tre forskellige bakteriearter: *Acinetobacter johnsonii*, *Escherichia coli* og *Citrobacter freundii*. Multi-resistensen hos de tre teststammer blev udnyttet i forsøgene ved tilsætning af de tre antibiotika som selektiv markør til påvisning af teststammerne blandt den mindre resistente normale bakterieflora. Ved forsøgene blev der tilsat lave kimtal af teststammerne (10^3 - 10^4 CFU ml^{-1}) til efterligning af de faktiske forhold når behandlet spildevand med multi-resistente bakterier udledes til akvatiske recipienter.

To ud af de tre multi-resistente teststammer, *Escherichia coli* og *Citrobacter freundii*, overlevede længere end 1 måned i saltvand i laboratorieforsøgene og i ferskvandsdammen. Derimod overlevede *Acinetobacter johnsonii* teststammen kun kort tid i begge forsøgsopstillinger. Resultaterne viste, at multi-resistente bakteriestammer som forekom i byspildevand kunne overleve relative lange perioder efter deres udledning i akvatiske recipienter. De multi-resistente bakterier overlevede bedre i sterilt (autoklaveret) vand, hvilket tyder på, at deres overlevelse blev påvirket af den tilstedeværende naturlige mikroflora. Sandsynligvis på grund af antagonisme eller predation. Ydermere, udviste teststammerne stadig multi-resistens efter 1 måneds ophold i ferskvandsdammen. Dette tydede på, at stress og næringsstof sult ikke påvirkede bakteriernes evne til at udvise antibiotikaresistens.

Muligheden for at overføre antibiotika resistensgener fra spildevand til bakterier i naturlige akvatiske miljøer blev undersøgt ved udspredning på agarmedier i laboratorieforsøg i projektets Del III. I alt 10 forskellige tetracyklin-resistente *Acinetobacter* bakterier isoleret i spildevand (n=10) blev udstreget på vækstmedium sammen med en tetracyklin-følsom *Acinetobacter* bakterie isoleret fra en å, som ikke var forurenet med spildevand. Kun to ud af de 10 donorbakterier var i stand til at overfører tetracyklin-resistens med den anvendte metode. I et af disse tilfælde var der en sammenhæng med overførsel af tetracyklin-resistens og overførsel af små plasmider fra donor til recipientbakterien, hvorimod overførsel af resistens i det andet tilfælde ikke var medieret af plasmid conjugation. Disse resultater indikerer, at antibiotika resistensgener i spildevand kan spredes til den naturlige akvatiske mikroflora. Det begrænsede antal anvendte teststammer, og det forhold at kun to donorbakterier var istand til at overføre resistens, tillader dog ikke en bestemmelse af omfanget af resistensoverførsel i naturlige akvatiske miljøer.

Effekten af udledning af byspildevand på spredning af antibiotika resistens blev yderligere undersøgt ved at sammenligne forekomsten af resistente bakterier i blåmuslinger fra områder hvortil der blev udledt spildevand og fra områder som ikke modtog spildevand. Blåmuslinger blev valgt som indikatorer på grund af deres høje bakterieoptag gennem daglig filtration af store vandmængder. Blåmuslinger eksponeret til behandlet spildevand indeholdt en højere procentdel ampicillin-resistente bakterier (12,9 til 95,5%), sammenlignet med blåmuslinger fra områder som ikke modtog behandlet spildevand (1,5 til 5,4%). Derimod var procentdelen af gentamicin og tetracyklin-resistente bakterier lav (<3%), uafhængig af muslingernes

oprindelse. Multi-resistente bakterier blev kun fundet i blåmuslinger eksponeret til behandlet spildevand og her i lave koncentrationer (#0.1%). Disse fund bekræfter, at behandlet spildevand kan være kilde til spredning af multi-resistente bakterier i akvatiske miljøer. Endvidere var der procentvis flere resistente bakterier i blåmuslinger indsamlet fra områder tæt på spildevandsudløbet sammenlignet med blåmuslinger indsamlet 100 m fra spildevandsudledningen. Dette indikerer en sammenhæng mellem forekomsten af resistente bakterier og afstanden til stedet for spildevandsudledning.

Konklusioner

Undersøgelsernes resultater viser, at forekomsten af enkelt og multi-resistente bakterier kan øges ved udledning af spildevand fra farmaceutiske virksomheder som producerer eller forarbejder antibiotika-holdige produkter. Disse undersøgelser syntes at være de første, som har påvist en sammenhæng med udledning af spildevand fra farmaceutiske virksomheder og forekomsten af resistente bakterier i spildevand. Den fundne stigning i prævalensen af resistente *Acinetobacter* kunne være forårsaget af resistente bakterier i virksomhedens spildevand og/eller udledning af antibiotika-holdigt spildevand som selekterede for resistente bakterier i kloakken. Disse fund viser, at spildevand fra farmaceutiske virksomheder sandsynligvis er vigtige kilder til forekomst og spredning af resistente bakterier i spildevand. Det skal dog bemærkes, at undersøgelserne kun blev udført ved en enkelt farmaceutisk virksomhed. Undersøgelser af yderligere virksomheder er derfor nødvendige til bestemmelse af omfanget af selektion og/eller introduktion af resistente bakterier gennem udledning af spildevand fra sådanne virksomheder.

Forekomst og overlevelse af resistente bakterier i spildevand bør vurderes varsomt på grund af muligheden for overførsel af disse bakterier mellem forskellige miljøer. Spildevand og kloakker er således egnede habitater for forekomst og spredning af resistente bakterier. Endvidere modtager og viderefører de spildevand fra en række kilder, hvorfra der udledes antibiotika og resistente bakterier (eksempelvis hospitaler, farmaceutiske virksomheder, landbrug og slagterier), til naturlige vandmiljøer. En egentlig risikovurdering var ikke opstillet som formål i vores undersøgelser. Men mulige sundhedsrisici for mennesker kan eventuelt være behæftet med spredning af resistente bakterier i spildevand, eksempelvis ved en eventuel forurening af drikkevand og badevand.

Vores undersøgelser viser, at spildevandsrensning medfører en reduktion i det totale antal resistente bakterier. Der fandtes ingen forskel i reduktionen af følsomme og resistente bakterier da den procentvise forekomst af resistente bakterier var ens i behandlet sammenlignet med ubehandlet spildevand. Spildevandsrensning ser således ud til nedsætte spredningen af resistente bakterier i spildevand.

Vores undersøgelser viste dog også at:

- Multi-resistente bakterier i ubehandlet spildevand kan overleve behandling og spredes til naturlige vandmiljøer gennem udledning af byspildevand.

- Multi-resistente bakterier i behandlet spildevand kan overleve i relativt lange perioder og bibeholde deres resistens egenskaber efter udledning til recipienten.

- Bakterier som var resistente overfor tre eller fire forskellige antibiotika blev isoleret i blåmuslinger fra områder som modtog behandlet spildevand. Derimod kunne sådanne multi-resistente bakterier ikke påvises i blåmuslinger og vandprøver fra områder som ikke modtog spildevand.

- Resistente bakterier i spildevand kan overfører antibiotika resistens til følsomme bakterier i ikke-forurenede akvatiske miljøer.

Resultaterne fra vores undersøgelser understreger behovet for at fastlægge i hvilket omfang spildevand spreder multi-resistente bakterier. Fremtidige studier bør undersøge forekomst og spredning af multi-resistente bakterier i byspildevand fremfor bakterier med resistens overfor et enkelt antibiotikum. Der er også behov for yderligere undersøgelser af multi-resistente bakteriers evne til resistensoverførsel til den normale akvatiske mikroflora under naturlige forhold. Endvidere bør spredningen af resistente bakterier og deres resistensgener efter udbringning af spildevandsslam på landbrugsjord undersøges. Endelig bør der foretages en vurdering af i hvilket omfang resistente bakterier i vandmiljøer bidrager til resistensproblemerne indenfor det veterinær og humane område.

1 Introduction and project background

This chapter describes the structure and objectives of the project, and provides the reader with the basic knowledge necessary to understand the problems addressed in the report. A description of the project is followed by a short introduction on antibiotics and antibiotic resistance, including an explanation of the problems occurring when antibiotic resistance is to be measured in bacterial populations. The public health and ecological concerns associated with the emergence of bacterial resistance are discussed. Finally, considerations are made concerning the importance of sewage in the spread of antibiotic resistance between different bacterial populations and environments.

1.1 Project structure and objectives

The project is composed of three parts, each part focusing on a particular feature concerning the occurrence and fate of resistant bacteria in sewage (Fig. 1). In the Part I, the effects caused by the discharge of waste effluent from a hospital and a pharmaceutical plant manufacturing antibiotics were investigated using *Acinetobacter* as a bacterial indicator. In the Part II, the effects of tertiary sewage treatment on total and relative numbers of resistant bacteria were monitored at two large-scale treatment plants for a period of six months. In the Part III, multiple-resistant strains isolated from treated sewage were analysed for their ability to survive in the aquatic environment. In addition, the impact of municipal sewage effluents on the spread of antibiotic resistance was further evaluated by studying the transfer of tetracycline resistance between *Acinetobacter* strains isolated from sewage and unpolluted freshwater, and by comparing the occurrence of resistant bacteria in shellfish exposed/non-exposed to sewage effluents.

Figure 1.1. Schematic representation of the project
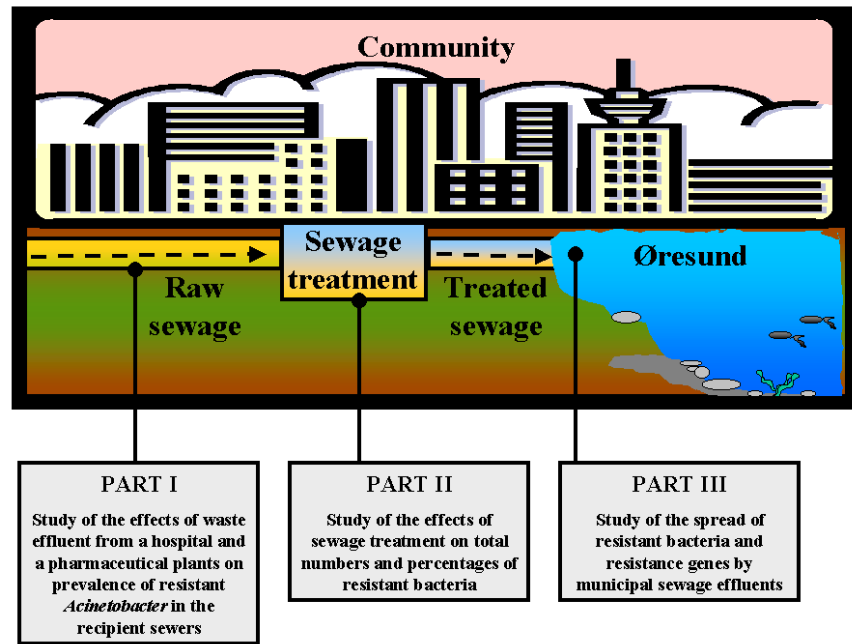
The general aim of the project was to identify factors influencing the occurrence of resistant bacteria in sewage and to study the fate of such bacteria along the sewage system. The assessment of the occupational risks associated with the occurrence of resistant bacteria in sewage treatment plants and the public health risks associated with the spread of multiple-resistant bacteria *via* municipal sewage effluents was not part of this study. The present work, however, represents a good basis for the development of future studies on risk assessment.

The following specific objectives were pursued as part of the project:

- To assess the effects of waste effluent from a hospital and a pharmaceutical plant on the prevalence of resistant *Acinetobacter* in the recipient sewers (Part I).

- To detect changes in the distribution of *Acinetobacter* strains/species associated with the discharge of waste effluent from these sources (Part I).

- To determine to what extent sewage treatment reduces the total numbers of resistant bacteria depending on the antibiotic, the bacterial population and the treatment plant under study (Part II).

- To evaluate the effects of sewage treatment on numbers of single and multiple-resistant bacteria (Part II).

- To determine the ability of multiple-resistant bacteria originating from treated sewage to survive in laboratory marine microcosms and in membrane diffusion chambers immersed in a freshwater pond (Part III).

- To demonstrate *in vitro* transfer of antibiotic resistance from bacteria isolated from sewage to related bacteria occurring in unpolluted aquatic environments (Part III).

- To determine whether differences in the number of resistant bacteria exist in shellfish from sites exposed to treated sewage and in shellfish from unpolluted sites (Part III).

1.2   What are antibiotics?

Antibiotics are substances produced by living organisms, which are able to kill or inhibit the growth of microorganisms. According to the literal sense of the word, substances produced synthetically (e.g. sulfonamides or quinolones) should not be termed antibiotics, and the use of a broader term (i.e. antimicrobial agent) would be more appropriate to indicate the complex of all substances having a harmful effect on microorganisms [1,1]. However, the term antibiotic is used throughout the present report as a synonym of antimicrobial agent.

Antibiotics are selectively toxic substances as they affect pathogenic microorganisms more adversely than the host. The degree of selective toxicity depends on the specific mechanisms of action of the drug. The most selective agents are those affecting structures (e.g. cell wall) or functions (e.g. folic acid synthesis) present only in prokaryotic cells. The less selective antibiotics are those affecting protein (e.g. tetracyclines) or nucleic acid synthesis (e.g. quinolones), which are essential functions for both prokaryotic (bacteria) and eukaryotic cells (the host). Among the antibiotic agents produced synthetically, are some that are toxic for humans and animals, and their use is restricted to inanimate objects (i.e. disinfectants) or the surface of living tissues (i.e. antiseptics). These agents are generally termed biocides.

1.2.1 Classification

Antibiotics are classified based on their chemical structure. Each class of antibiotics is characterised by a typical core structure and the various members of the class are differentiated by the addition or subtraction of secondary chemical structures from the core structure. The main classes of antibiotics currently used in clinical practice include penicillins, cephalosporins, tetracyclines, aminoglycosides, fluoroquinolones, potentiated sulfonamides, macrolides and glycopeptides.

Antibiotics can also be classified as broad, intermediate or narrow spectrum, depending on the range of bacterial species against which they are active [1]. Broad-spectrum antibiotics include compounds active against both Gram-positive and Gram-negative bacteria like quinolones, tetracyclines, and third generation cephalosporins. Intermediate spectrum antibiotics generally include substances with reduced activity against some Gram-negative bacterial species (e.g. ampicillin, amoxicillin, first and second generation cephalosporins). Narrow spectrum antibiotics are only effective against restricted groups of bacteria. For example, penicillin is only active against Gram-positive bacteria, whereas, aminoglycosides, sulfonamides and trimethoprim are solely active against aerobic bacteria [1].

1.2.2 Mechanisms of action

Antibiotics constitute quite a heterogeneous group of chemicals. Depending on the chemical structure, antibiotics exert an effect on different structures or functions of the bacterial cell (Fig. 1.2). The major mechanisms of action are inhibition of the cell wall synthesis (e.g. penicillins and vancomycin), damage of the cell membrane function (e.g. polymixins), inhibition of protein synthesis (e.g. aminoglycosides, tetracyclines, chloramphenicol, lincosamides and macrolides), inhibition of the nucleic acid synthesis (e.g. quinolones and rifampicin), and metabolic antagonism (e.g. sulfonamides and trimethoprim)[1].

Figure 1.2. Sites of action for selected antibiotics. PABA, para-aminobenzoic acid; DHFA, diydrofolic acid; THFA, tetrahydrofolic acid. Modified from Prescott and Baggot [1].
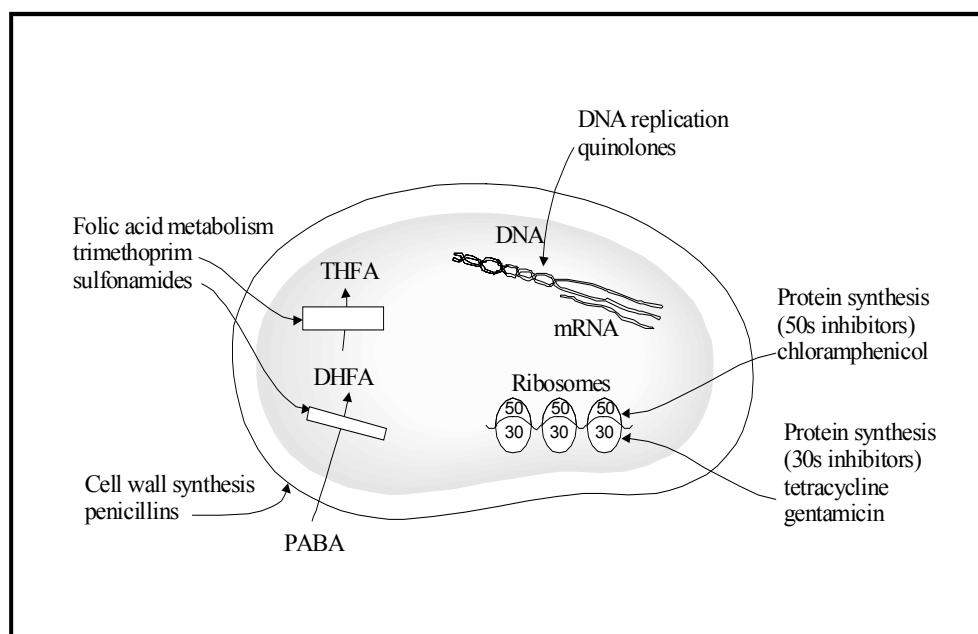
1.3   What is antibiotic resistance?

Antibiotic resistance is a relative term. A bacterial strain can be defined resistant if it survives in the presence of higher antibiotic concentrations in comparison with phylogenetically related strains [2]. Thus, antibiotic resistance is not a bacterial property that can be determined by studying a single strain, but only by comparison under identical conditions of two or more strains belonging to the same genus or species.

The above-mentioned definition of antibiotic resistance refers to *in vitro* conditions. Under *in vivo* conditions, antibiotic resistance is a context-dependent term as it depends on the location of the bacterium and the bioavailability of the drug. For example, bacteria are less susceptible to antibiotics when assembled in biofilms (complex communities of microorganisms embedded in a matrix of extracellular material) compared with the same organisms living separately [3]. In aquatic environments, binding of the antibiotic molecule with ions or substances present in sediment strongly reduces both the activity of the drug and its absorption in the fish intestine [4].

1.3.1 Molecular mechanisms

Bacterial resistance to antibiotics can be caused by different molecular mechanisms [5]. The most common mechanisms include: reduced drug uptake (e.g. membrane impermeability to cephalosporins); active drug efflux (e.g. tetracycline efflux pumps); drug deactivation (e.g. hydrolysis of penicillins by beta-lactamases), modification of the drug target (e.g. mutations of the DNA gyrase leading to quinolones resistance); increased concentration of the drug target (e.g. increased folic acid production that counteracts the inhibition of such production by sulfonamides), or alternative pathways to elude the drug effect (e.g. synthesis of folic acid using an enzyme which is not affected by sulfonamides) (Fig. 1.3).

Figure 1.3. Molecular mechanisms of antibiotic resistance. Modified from Hayes and Wolf [5].
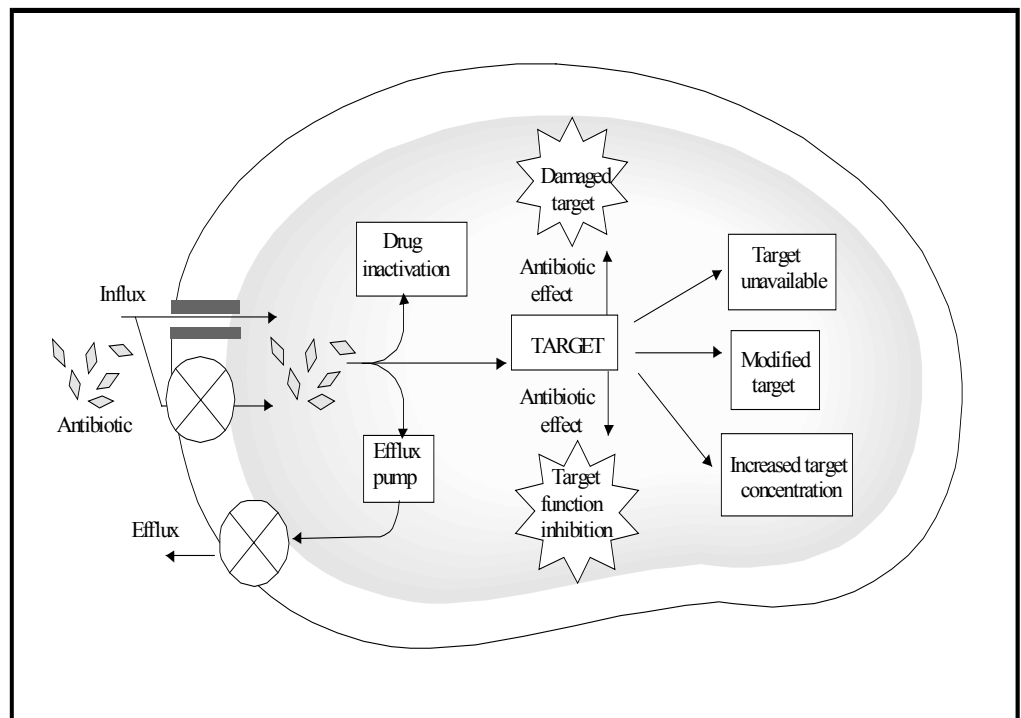
1.3.2 Natural and acquired resistance

An important distinction should be made between natural and acquired resistance. Bacteria are termed naturally, intrinsically or constitutively resistant when resistance is due to characteristic features typical of the species. For example, *Pseudomonas aeruginosa* is naturally resistant to penicillins, due partly to the inability of the drug to diffuse through the outer membrane [6] and partly to the deactivation of the drug by chromosomally encoded enzymes (i.e. beta-lactamases) [7].

In contrast, acquired resistance emerges in a bacterial population that was previously susceptible, because of modifications of the bacterial DNA caused by either chromosomal mutation or horizontal gene transfer. Natural resistance results from a long process of genetic evolution, whereas, acquired resistance can arise within a short time (one or few generations) [5].

1.3.3 Acquisition by chromosomal mutations

Mutation is a heritable change in the sequence of the DNA occurring due to errors during DNA replication [8]. The frequencies of chromosomal mutations leading to antibiotic resistance depend on the specific antibiotic. For example, mutation frequencies are high for compounds like nalidixic acid, rifampicin and streptomycin (10^{-8} to 10^{-10} cells per generation), low for erythromycin and are not known to occur for vancomycin and polymixin B [9]. For antibiotics like streptomycin, a single mutation can determine a 1000-fold increase in the resistance levels [9]. In contrast, for other drugs (e.g. quinolones) the acquisition of resistance is a gradual, step-wise process in which different mutations are involved [10].

1.3.4 Acquisition by horizontal gene transfer

Horizontal gene transfer is the relocation of genetic material from one bacterial cell (donor) to another (recipient). Such a transfer may occur directly by physical contact (*conjugation*) or indirectly, using the surrounding medium (*transformation*) or bacteriophage (*transduction*) as vectors [11](Fig. 1.4). Bacterial transfer of antibiotic resistance has been demonstrated to occur in various natural habitats, including water, sediment, soil, plants and animals [12,13]. The DNA transferred from the donor to the recipient may be contained in mobile genetic elements called plasmids, structures of circular DNA that reproduce independently from the chromosome [11]. Unlike chromosomes, plasmids generally do not encode functions essential to bacterial growth, but functions that are of importance under particular conditions, such as antibiotic resistance, heavy metal resistance, metabolic functions, or production of antibiotics, toxins and virulence factors [14].

1.3.5 Intracellular migration of resistance genes

Antibiotic resistance genes can migrate from one site to another on the bacterial genome using small vectors called transposons [15] and integrons [16]. These genetic elements containing antibiotic resistance genes are able to move between different sites of the bacterial genome without any requirement of DNA homology. This process is known as non-homologous recombination (to a site that does not match with the gene) and differs from the normal process of genetic recombination, which requires a high degree of DNA homology (a near perfect match) [11,17]. Both transposons and integrons make it possible for new antibiotic resistance genes to be acquired by plasmids and subsequently spread in the bacterial population by mechanisms of horizontal gene transfer, as suggested by the frequent recovery of these genetic elements as part of broad host plasmids [18,19].

Figure 1.4. Mechanisms of bacterial genetic transfer (Levy [20]).
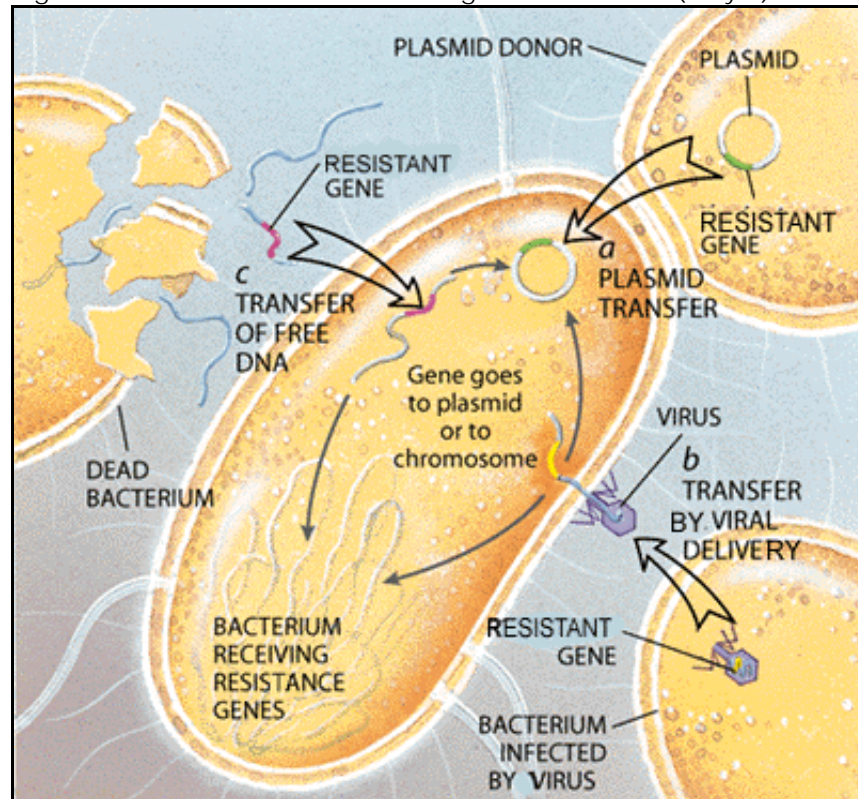
1.3.6 Measurement of resistance in bacterial populations

The value of the term "measurement of antibiotic resistance" in environmental microbiology generally differs from that in clinical studies. The main concern for environmental microbiologists is to investigate the distribution of antibiotic resistance in bacterial populations rather than the level of resistance in individual strains. Unfortunately, culture methods are not efficient enough to determine the actual prevalence of antibiotic resistance in a bacterial population. In fact, only a small proportion of the aquatic bacterial flora (<1%) can be cultured on laboratory media [21].

The method traditionally used for the measurement of antibiotic resistance at the population level consists in standard bacteriological counts on media containing specific concentrations of antibiotics. The main drawback of this method is the use of a single breakpoint for the determination of antibiotic resistance. In fact, the use of a single breakpoint, corresponding to the amount of antibiotic agent added to the medium, does not take into account the variability in the levels of antibiotic resistance existing among different bacterial species. Consequently, bacteria characterised by intermediate levels of resistance can be classified either as resistant or susceptible depending on the concentration of antibiotic added to the medium.

An alternative approach is to use a group of phylogenetically related organisms as bacterial indicators of antibiotic resistance. This method is based on the principle that spatial and temporal differences observed in the levels of antibiotic resistance of the bacterial indicator are indicative of the selective pressure to which the entire bacterial population is exposed. Thus, this method does not aim to determine the exact prevalence of antibiotic resistance

in the bacterial population under study, but rather to detect the effect of potential sources of antibiotic resistance on the bacterial population.

The use of bacterial indicators offers various advantages compared with bacteriological counts on antibiotic selective media. The isolation and identification of bacteria makes possible the use of standardised methods for antibiotic susceptibility testing, namely the disc-diffusion method and the dilution method. When antibiotic susceptibility testing is performed on a large number of bacterial isolates, results can be used to understand the distribution of antibiotic resistance within the target bacterial population and consequently to define appropriate breakpoints for the classification of resistant and susceptible strains.

Today, the nucleotide sequences of many genes encoding for antibiotic resistance are available. DNA-DNA hybridisation and PCR methods are currently used to investigate the presence of resistance genes in environmental bacteria. In comparison with phenotypic methods, genetic methods offer the great advantage to investigate also non-culturable bacterial species. However, the currently available genetic methods are only able to quantify, to a limited degree, the presence of a gene in a bacterial population. Furthermore, since a number of different genes can encode resistance to the same antibiotic agent, genetic methods cannot be used for quantitative assessment of resistance.

1.4 The microbial threat

In the last decades, bacterial resistance to antibiotics has assumed an increasing importance with regard to its impact on both public health and ecology. Obviously, the primary problem is represented by the emergence of antibiotic resistance among bacteria pathogenic to humans and animals, which makes difficult the treatment of some life-threatening infections. However, independent from the risks for human health, is the spread of antibiotic resistance and the problems raised in ecological nature. In fact, the introduction and selection of resistant bacteria in the environment can lead to structural changes in the composition of microbial communities, with possible deleterious effects on the balance of natural ecosystems.

1.4.1 The emergence of resistance in human pathogenic bacteria

In the past, bacteria were the most important cause of disease and mortality among humans. The introduction of antibiotics in human medicine has markedly reduced the impact of bacterial diseases on human mortality. Nevertheless, the extraordinary capacity for adaptation of bacteria soon allowed these organisms to develop mechanisms of resistance enabling them to overcome the toxic effects of antibiotics.

A survey on enterobacterial isolates collected between 1917 and 1954 has demonstrated that bacteria were generally susceptible to antibiotics before these drugs became commonly available in human medicine [22]. However, other studies indicate that resistant bacteria were present at the time, although they were not prevalent in bacterial populations [23]. Thus, it appears that the indiscriminate use of antibiotics has played a major role in the emergence of antibiotic resistance by exerting a selection in favour of resistant bacteria.

The first case of penicillin resistance in *E. coli* was reported in the 1950's. Since then, things have taken a turn for the worse. Today, antibiotic resistance

represents an important problem in the therapy of various human pathogenic bacteria. Three bacterial species causing life-threatening infections (*Pseudomonas aeruginosa*, *Mycobacterium tubercolosis* and *Enterococcus faecalis)* can demonstrate resistance to any available antibiotic [20]. Vancomycin is the only effective drug for treatment of infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA), but the occurrence of strains with reduced susceptibility to this antibiotic has already been reported [24]. Problems may also occur in the therapy of hospital infections caused by *Acinetobacter baumannii*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Neisseria meningitidis* and *Streptococcus pneumoniae* [20].

The problem of antibiotic resistance is of particular concern for immunosuppressed patients, suchas those affected by HIV, cancer or chronic diseases, as antibiotic therapy represents the only way to overcome bacterial infections for these people. Serious problems may also occur in developing countries where the use of new and expensive drugs is limited by their cost and availability. In addition to the risks for human health, this situation incurs a worldwide increase in the cost of hospital care, including the use of new expensive drugs, increased costs for bacteriological analysis and prolonged hospitalisation [25].

1.4.2 The spread of resistance among environmental bacteria

Antibiotic resistance is not only found in pathogenic bacteria but also in environmental organisms inhabiting terrestrial and aquatic habitats. The occurrence of resistant bacteria in nature may have originated from antibiotic-producing organisms, as suggested by the evidence that in some cases the mechanisms and genes protecting these organisms from the antibiotics they produce are similar to those responsible for resistance in clinical isolates [26]. However, higher numbers of resistant bacteria occur in polluted habitats compared with unpolluted habitats [27,28], indicating that humans have contributed substantially to the increased proportion of resistant bacteria occurring in the environment.

Possible mechanisms by which humans enhance the spread of antibiotic resistance among environmental bacteria include the deliberate or accidental introduction of antibiotics, resistant bacteria and resistance genes into the environment. Antibiotics exert a selection in favour of resistant bacteria by killing or inhibiting growth of susceptible bacteria (see section 1.5.1); resistant bacteria can adapt to environmental conditions and serve as vectors for the spread of antibiotic resistance; resistance genes can be taken up by indigenous bacteria and spread by mechanisms of genetic transfer (see section 1.3.4).

The main risk for public health is that resistance genes are transferred from environmental bacteria to human pathogens. The ability of resistant bacteria and resistance genes to move from one ecosystem to another is documented by the various cases in which transmission of resistant bacteria has been demonstrated between animals and humans [29,30]. The inclusion of certain growth promoters in animal feed has been recognised as a cause for the selection of resistance genes in the commensal microflora of animals and their transmission to humans *via* the food chain [29,30]. Similarly, drinking and bathing water could represent a source for the acquisition of resistant bacteria in humans. However, further studies are necessary to validate this hypothesis.

The ecological consequences associated with the dissemination of resistant bacteria in the environment have been scarcely investigated. However, it

appears evident that environmental contamination with antibiotics, resistant bacteria and resistance genes affects the biodiversity of natural ecosystems. Antibiotics are likely to determine a reduction in the levels of microbial diversity by the suppression of susceptible organisms, including bacteria, fungi, protozoa and algae. Resistant bacteria and genetic elements could find favourable conditions to become predominant in habitats contaminated by antibiotics, thereby, altering the original composition (balance) of natural microbial communities.

1.5   Spread of antibiotic resistance in sewage

Sewage is waste matter resulting from the discharge into the sewers of human excreta and wastewater originating from the community and its industries. Sewage contains a high content of both organic and inorganic matter, as well as high densities of living organisms, including pathogenic, commensal and environmental bacteria. This chacteristic composition makes sewage a particularly suitable ecological niche for the growth and spread of antibiotic resistance.

1.5.1 Antibiotic selective pressure

The acquisition of antibiotic resistance genes is generally independent of the presence of antibiotics. However, the exposure of bacteria to antibiotics confers an ecological advantage to resistant strains on susceptible strains, allowing them to become predominant in the bacterial population. This situation is commonly termed as *antibiotic selective pressure* and can occur in either the host *in vivo* (eg. human or animal body) as a consequence of chemotherapy or in the environment, for example when antibiotic residues are introduced in sewage.

Residues of antibiotics administered to humans and animals reach the sewage systems in urine or faeces, in the form of either parent compound or degraded metabolites depending on the pharmacology of the specific antibiotic. Furthermore, an unknown amount of antibiotics enter the sewers by waste derived from antibiotic production and disposal of a surplus of drugs. Indeed, various antibiotics have been found in municipal sewage, including fluoroquinolones, sulfonamides and erythromycin metabolites [31-34]. The antibiotic concentrations found in sewage vary between 1 and 100 μg per liter. Such concentrations are 100- to 1000fold lower compared with those necessary to inhibit resistant bacteria, but are sufficient to affect susceptible bacteria [35,36]. Therefore, the occurrence of such antibiotic concentrations in sewage has the potential to select for antibiotic resistance.

The fate of antibiotics in sewage depends on their chemical properties. Lipophilic and non-readily degradable substances are likely to be retained in the sludge, whereas, hydrophilic substances may be able to pass through treatment plants and end up in the natural recipients receiving treated sewage [37]. It also appears that the solubility in water of drug metabolites is generally higher compared with the parent compounds [37]. Thus, it is likely that a large proportion of the antibiotic residues introduced into the sewage system can reach surface waters through municipal sewage effluents.

1.5.2 Non-antibiotic selective pressure

Among the multitude of substances occurring in sewage, there are some that have the potential to select for antibiotic resistance, even though they are not antibiotics. Heavy metals and biocides are two important groups of non-antibiotic substances showing this property. Heavy metals are widespread in sewage as a consequence of industrial pollution. Biocides are introduced into sewage by hospitals, farms, slaughterhouses and food-processing establishments; where these agents are used for the disinfection of environments and utensils, or by the community, due to the presence of these agents in house-hold products, such as soaps and dishwashing detergents.

There are two possible ways by which heavy metals and biocides can select for antibiotic resistance. The genes encoding resistance to heavy metals and biocides can be located together with antibiotic resistance genes on either the same genetic structure (e.g. plasmid), or different genetic structures within the same bacterial strain. Alternatively, bacteria can have unspecific mechanisms of resistance to different substances, including heavy metals, biocides and antibiotics. In both cases, exposure to one substance results in the selection of bacterial strains able also able to resist the other substance (*co-selection*).

Genes encoding resistance due to heavy metals and antibiotics often co-exist on plasmids [38]. In addition, unspecific mechanisms conferring resistance to both heavy metals and antibiotics are known to exist in some bacterial species (e.g. active pump-efflux system encoded by the *marA* gene in *E. coli*). The co-selective property of heavy metals is confirmed by the indirect evidence that bacteria isolated from heavy metal-polluted marine sediment are significantly more resistant to antibiotics compared with bacteria isolated from unpolluted sites [39].

Although genes encoding resistance to biocides have been found on plasmids and integrons [40], these substances are more likely to select for antibiotic resistance by induction of unspecific mechanisms of multiple resistance. Laboratory experiments have shown that biocides such as triclosan and pine oil can select for resistance to different antibiotics when bacteria are exposed to low concentrations of biocide [41,42]. Accordingly, the co-selective effect of biocides for antibiotic resistance could be particularly marked when these substances are dispersed in the environment, because of dilution and formation of concentration gradients.

1.5.3 Optimal conditions for horizontal gene transfer

Sewage is a suitable habitat for the transfer of resistance genes across different groups of bacteria. In this habitat, environmental bacteria meet resistant bacteria selected by use of antibiotics in human and veterinary medicine. Consequently, resistance genes occurring in bacteria of human and animal origin can be transferred to environmental bacteria, contributing to the formation of an environmental pool of resistant bacteria and resistance genes.

The high concentrations of bacteria, nutrients and suspended solids in sewage are all factors enhancing horizontal gene transfer [43-45]. High bacterial concentrations increase the chance that donor and recipient cells come in contact. Nutrients are more likely to have an indirect influence on the occurrence of gene transfer by increasing the concentration and the metabolic activity of bacteria. Suspended solids provide ideal surfaces on which the

various components contributing to the process of horizontal gene transfer (bacteria, free DNA and bacteriophages) are concentrated.

Plasmids and transposons harbouring antibiotic resistance genes are widespread in the bacterial flora of sewage [46,47]. Multiple-resistant bacteria isolated from sewage can transfer plasmid-mediated antibiotic resistance at high frequencies in the laboratory [48,49]. Experiments performed using membrane chambers immersed in sewage have shown that high frequencies of transfer may also occur under real conditions [50,51].

2 Methodology

2.1 Sampling sites, times and methods

As part of the study samples of sewage were collected from sewers and sewage treatment plants. The following two sections detail the sites sampled, sampling times and methods used.

2.1.1 Sampling at sewers

Samples of sewage were collected from two separate sewers receiving waste effluent from a hospital and a pharmaceutical plant manufacturing products containing antibiotics (Paper 1). Sampling sites were situated upstream (site A) and downstream (sites B and C) from the effluent discharge points of the hospital and the pharmaceutical plant (Fig. 2.1). At the hospital, site A was situated 350 m upstream from the discharge point, site B was 60 m downstream and site C was 500 m downstream. At the pharmaceutical plant, site A was located 70 m upstream from the discharge point, site B was 30 m downstream and site C was 250 m downstream.

The selection of the sampling sites was restricted by the access to the sewer system for the collection of samples. No important sources of waste effluent were present between sites A and sites B. Therefore, differences in the occurrence of resistant bacteria between these sites could be used to evaluate the impact associated with the discharge of waste effluent.

Samples were collected between June and September 1997, for a total of four sampling times. Samples of sewage mixed with sediment particles were aspirated from the bottom of sewers using sterile catheters applied to 150 ml sterile syringes. Samples were delivered to the laboratory within one hour after their collection.

Figure 2.1. Schematic representation of the sampling sites at the sewers receiving waste effluent from the hospital (A) and the pharmaceutical plant (B)
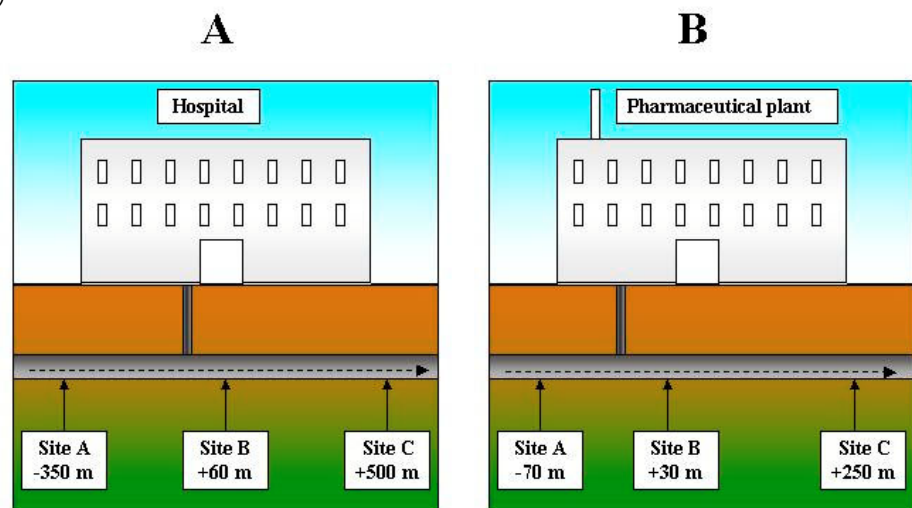
2.1.2 Sampling at sewage treatment plants

Samples of raw sewage, treated sewage and anaerobically digested sludge were collected at two large-scale treatment plants in Denmark, Avedøre Spillevandscenter I/S and Lynettefællesskabet I/S (Paper 4). The catchment areas of the two plants have a population of approximately 240,000 and 500,000 inhabitants, respectively. At both plants, sewage undergoes tertiary (advanced) treatment. The treatment process includes: retention of large solids by mechanical screens; separation of sand, grit and grease in aerated chambers; sludge sedimentation in primary settling tanks; biological removal of nitrogen and phosphorus by activated sludge units (supplemented by chemical phosphorus precipitation), and secondary clarifiers.

Sampling was performed monthly from August 1999 to January 2000, for a total of six sampling times at each plant. Twenty-four hour flow-proportional samplers were used to collect raw sewage from the influent, and samples of treated sewage from the final effluent. Grab samples of digested sludge were collected from the digesters following centrifugation. All samples were processed in the laboratory within 6 hours of their collection.

2.2   Measurement of antibiotic resistance

Two different methods were used for measurement of antibiotic resistance of bacteria in sewage samples. *Acinetobacter* isolates were randomly isolated without inclusion of antibiotics in the medium (non-selective method) and the prevalences of antibiotic resistance were evaluated based on antibiotic susceptibility testing of large numbers of isolates. In addition, total and relative numbers of resistant bacteria were calculated based on bacteriological counts on media containing antibiotics. The latter method differs from the former, in that bacteria are selectively isolated with regard to their antibiotic resistance properties (selective method).

2.2.1 Use of Acinetobacter as a bacterial indicator

One of the main innovative aspects of this project was the use of *Acinetobacter* as a bacterial indicator. In fact, most previous studies investigating the occurrence of resistant bacteria in sewage were performed using coliforms as bacterial indicators. The choice of *Acinetobacter* was prompted by the natural occurrence of this organism in the aquatic environment. Based on this characteristic, *Acinetobacter* was considered a more representative indicator of the aquatic bacterial flora in contrast to coliforms, which occur in sewage mainly through human and animal contamination.

In the last decade, *Acinetobacter* has assumed an increasing importance as an opportunistic human pathogen, causing infections often refractory to antibiotic treatment [52]. The particular ability of *Acinetobacter* to develop antibiotic resistance suggested us that this organism that could be used as a sensitive indicator for monitoring antibiotic resistance. Furthermore, *Acinetobacter* represents a good model for studying the ecology of antibiotic resistance genes, as it occurs ubiquitously in a large variety of habitats, including water, soil, food, animals and humans (201).

Several practical features also contributed to the choice of *Acinetobacter* as a bacterial indicator. *Acinetobacter* is a non-fastidious organism in the laboratory

and therefore allows antibiotic susceptibility testing by standardised procedures. Furthermore, the availability of a pre-established selective enrichment medium and a genus-specific DNA probe allowed us to develop a rapid method for isolation and identification (see section 2.3.1). This aspect was of primary importance in the choice of the bacterial indicator, as large numbers of isolates are required for statistically validating data on antibiotic resistance.

2.2.2 Antibiotic susceptibility testing of Acinetobacter isolates

Antibiotic susceptibility testing of *Acinetobacter* isolates was performed by the disc-diffusion method in accordance with the Swedish Reference Group for Antibiotics [53]. All isolates were tested against antimicrobial compounds representative of six antibiotic classes: amoxicillin or ampicillin (penicillins), chloramphenicol (phenicols), ciprofloxacin (quinolones), gentamicin (aminoglycosides), tetracycline or oxytetracycline (tetracyclines), and sulfamethoxazole (sulfonamides) or sulfamethoxazole/trimethoprim (potentiated sulfonamides). *Acinetobacter* isolates from the two sewage treatment plants were additionally tested against aztreonam (monocyclic beta-lactam), cefoxitin (2nd generation cephalosporin), cefotaxime (3rd generation cephalosporin), imipenem (carbapenem), nalidixic acid (older quinolone), piperacillin (piperazine-penicillin), amikacin and tobramycin (aminoglycosides).

The breakpoints for classification of resistant and susceptible isolates were empirically selected based on the distribution of the inhibition zone diameters. The selection of the breakpoints was facilitated by the demonstration of two distinct sub-populations constituting resistance and susceptibility. In most cases, the elected breakpoints did not differ significantly from those used for defining resistance in clinical *Acinetobacter* isolates. In the few instances where they did not correlate, both empirical and clinical breakpoints were used (Paper 4).

2.2.3 Enumeration of resistant coliforms in sewage

Bacteriological counts of total and resistant coliforms were performed by the streak plate method. Total coliforms were enumerated on MacConkey agar following 24 hours incubation at 37°C, without confirmation of presumptive coliforms for gas production. Resistant coliforms were enumerated on the same medium containing ampicillin (16 μg/ml), gentamicin (8 μg/ml), tetracycline (8 μg/ml), or all three antibiotics using the same incubation conditions. The percentages of antibiotic resistance were then calculated for each sample as the number (CFU/ml) of resistant coliforms divided by the number of total coliforms.

Ampicillin, gentamicin and tetracycline were selected as representatives of important classes of antibiotics: beta-lactams, aminoglycosides and tetracyclines, respectively. The antibiotic concentrations added to the medium were in accordance with the minimum inhibitory concentration (MIC) breakpoint values for definition of resistance in clinical practice [53].

2.2.4 Enumeration of resistant acinetobacters in sewage

Total numbers and percentages of resistant acinetobacters (i.e. presumptive *Acinetobacter* spp. not identified by the genus-specific probe) in sewage were

determined as described above, with the exception of the agar (Baumann agar) and the incubation conditions (48 hours at 30°C) used.

2.2.5 Enumeration of total culturable resistant bacteria in blue mussels

Blue mussels were homogenised in a stomacher (5 g blue mussels in 10 ml sterile water for 30 s) and the obtained homogenate serially diluted ten-fold. Total numbers and percentages of culturable antibiotic-resistant bacteria were then determined as described above, with the exception of a different agar (Mueller-Hinton agar) and different incubation conditions (48 hours at 30°C). Mueller-Hinton agar was considered particularly suitable for enumeration of total culturable resistant bacteria, as it does not contain substances that could adversely affect the activity of antibiotics incorporated and permits satisfactory growth of most culturable bacterial species.

2.2.6 Enumeration of resistant *E. coli* in blue mussels

Total numbers and percentages of antibiotic-resistant *E. coli* in blue mussels were determined as detailed above, with the exception of a different agar (tryptone bile agar with X-glucoronide) and different incubation conditions (24 hours at 44°C).

2.2.7 Statistical analysis

Statistical analysis of data on antibiotic resistance was performed using either Statistix (Analytical Software, USA) (Paper 1) or SAS version 6.12 (SAS Institute Inc., USA) (Paper 4). The analyses were used to determine any statistically significant associations between antibiotic resistance (outcome variable) and sampling sites (independent variable), including the variable sampling time to allow for any confounding effect of time.

Data derived from antibiotic susceptibility testing of *Acinetobacter* isolates (dichotomous data) were analysed by logistic regression analysis. When such analysis was not appropriate due to excessive variability of the data, chi-square analysis was performed separately for each sampling time (Paper 1). Data derived from bacteriological counts (continuous data) were analysed by linear regression analysis, after testing whether the residuals were normally distributed.

In the study relating to the effects of waste effluent from the hospital and the pharmaceutical plant, comparisons between sites A and B were carried out to determine whether the discharge of waste effluent was associated with an increase in the occurrence of resistant isolates. Comparisons between sites B and C were carried out to provide information concerning variations in the prevalence of resistant *Acinetobacter* depending on the distance from the discharge point.

In the study concerning the effects of sewage treatment, the statistical analysis was performed separately for each plant. Data pertaining to raw and treated sewage were compared to assess the effect of sewage treatment on the prevalence of resistant bacteria. Data concerning raw sewage and digested sludge were compared to assess the effect of sludge treatment on the prevalence of resistant bacteria.

2.3 Identification and typing of bacteria and resistance genes

During the project, bacteria were characterised at various levels using phenotypic and genotypic methods. *Acinetobacter* isolates were identified at the genus level prior to antibiotic susceptibility testing. A number of isolates was characterised by phenotypically and their plasmid content determined to detect possible effects on strain distribution caused by the discharge of waste effluent from the pharmaceutical plant. Ribotyping was used to confirm the identity of strains introduced into membrane diffusion chambers during the *in situ* experiment on survival of multiple-resistant bacteria. Finally, tetracycline resistance genes were also typed to determine whether the same classes of resistance genes occurred in both clinical and aquatic *Acinetobacter* strains.

2.3.1 Identification of *Acinetobacter* at the genus level

Acinetobacter isolates were identified at the genus level by colony hybridisation using a genus-specific DNA probe. In order to enhance the detection of *Acinetobacter*, colony hybridisation was performed in combination with the use of the Baumann medium, which is a selective medium based on the ability of *Acinetobacter* to grow using acetate as the only carbon source [54]. The protocol used for colony hybridisation is described in Paper 1.

2.3.2 Identification of *Acinetobacter* at the species level

A subset of *Acinetobacter* isolates from the sewers at the hospital and the pharmaceutical plant (n=43) was identified at the species level by phenotypic tests. The following tests were performed: growth at 37℃ in Brain Hearth Infusion broth (BHI), acidification of glucose, haemolysis of sheep blood, utilisation of citrate, azelate, glutarate, L-histidine, DL-lactate, L-leucine, L-phenylalanine and L-arginine. The methods used for each test and the criteria used for identification are described in Paper 2.

2.3.3 Plasmid profiles

The same subset of *Acinetobacter* isolates from the sewers at the pharmaceutical plant and the hospital was characterised by plasmid profiling. Plasmids were isolated by a hot alkaline method [55] modified by the addition of lysozyme, and then detected by gel electrophoresis in 0.8% agarose gels.

2.3.4 Ribotyping

Due to its high discriminatory power, ribotyping was considered particularly suitable to confirm the identity of strains inoculated into membrane-filter chambers during the performance of the *in situ* pond experiment (see section 2.5.3).
Ribotyping was performed as previously described [56] using the restriction enzyme *Hin*d III for DNA digestion. The method entails digestion of bacterial DNA by restriction enzymes followed by DNA hybridisation with rRNA-based probes.

2.3.5 Typing of tetracycline resistance genes

Fifty tetracycline-resistant *Acinetobacter* isolates from clinical specimens (n=35), sewage (n=10) and aquaculture habitats (n=5) were analysed by PCR for the occurrence of tetracycline resistance genes of classes A to E, which are the predominant classes among Gram-negative bacteria. The PCR

primers and protocols used for the typing of tetracycline resistance genes are described in Paper 3.

2.4 Experiments on transfer of tetracycline resistance

In vitro experiments on the transfer of tetracycline resistance between *Acinetobacter* strains isolated from different environments and belonging to different species were carried out to assess their ability to transfer tetracycline resistance genes. We decided to focus our attention on tetracycline resistance due to the frequent occurrence of this resistance observed in *Acinetobacter* isolates originating from sewage and aquaculture habitats. Furthermore, tetracycline resistance was considered particularly suitable for studying the ability to transfer antibiotic resistance among aquatic bacteria as it is usually mediated by genetic transfer and only rarely determined by chromosomal mutations.

2.4.1 Bacterial strains

Twenty tetracycline-resistant *Acinetobacter* strains were used as donors in the mating experiments, including 10 strains from sewage, 5 strains from aquacultural habitats and 5 strains resulting from clinical outbreaks. The five clinical strains belonged to the species *A. baumannii* and originated from different European countries. Most aquatic strains (8/15) were identified phenotypically as *A. lwoffii* and *A. johnsonii*, the two species prevalent in the aquatic environment. The remaining strains belonged to *A. junii* (n=3), *A. haemolyticus* (n=1), the unnamed genomic species 16BJ (n=1) or had atypical phenotypic traits precluding speciation (n=2).

Rifampicin-resistant mutants obtained by the gradient plate method [57] were used as recipients. These were strains derived from tetracycline-sensitive *Acinetobacter* strains isolated from an unpolluted stream (recipient A) and sewage (recipients B and C). According to phenotypic identification, recipients A and B belong to unknown *Acinetobacter* species and recipient C belongs to *A. calcoaceticus*.

2.4.2 Mating experiments

Mating experiments were performed on solid media (Luria Bertani agar) as described in Paper 3. The acquisition of tetracycline resistance by recipient strains was confirmed by phenotypic tests differentiating between donors and recipients. Plasmid profiling was used to detect relocation of plasmid DNA from donor to recipient strains. Mating experiments in which transfer of tetracycline resistance was demonstrated, were studied to determine whether transfer was mediated by conjugation or transformation. In order to exclude transfer mediated by transformation, experiments were repeated in the presence of deoxyribonuclease I, an enzyme destroying extracellular DNA. In addition, the ability of the recipient strains to acquire resistance by transformation was tested by experiments in which DNA extracted from the donor strains was used as a source of resistance genes.

2.5   Experiments on survival of multiple-resistant bacteria in natural waters

Three multiple-resistant strains isolated from treated sewage were investigated for their ability to survive in natural waters and retain antibiotic resistance. This was tested using laboratory seawater microcosms and membrane-filter chambers immersed in a pond. The multiple resistance phenotypes characteristic of these strains were used as selective markers for their detection in the presence of indigenous bacteria. The experiments were performed using low bacterial inoculums (10^3 to 10^4 CFU/ml) with the scope to reproduce the actual conditions occurring when treated sewage is released into natural aquatic recipients.

2.5.1 Bacterial strains

Three multiple-resistant strains previously isolated from the final effluent of the Lynetten treatment plant, were used for the survival experiments. The strains were identified phenotypically as *Acinetobacter johnsonii* (strain B1), *Escherichia coli* (strain M1) and *Citrobacter freundiii* (strain M2) by the API identification system (Biomerieux, France). All three strains were resistant to ampicillin, gentamicin and tetracycline. In addition, strain B1 was resistant to tobramycin, nalidixic acid, potentiated sulfonamides, piperacillin and aztreonam. Strain M1 was resistant to nalidixic acid, ciprofloxacin and intermediate resistant to chloramphenicol. Strain M2 was resistant to chloramphenicol, potentiated sulfonamides and cefoxitin.

The strains were detected using Baumann agar (strain B1) and MacConkey agar (strains M1 and M2) containing ampicillin (16 µg/ml), gentamicin (8 µg/ml) and tetracycline (8 µg/ml). No bacterial growth was observed when these media were inoculated with either seawater or pondwater used for the survival experiments, indicating that bacteria with this multiple resistance phenotype were not present in the indigenous microflora.

2.5.2 Laboratory seawater microcosms

The first survival experiment was performed in microcosms containing seawater collected from Øresund. Microcosms (n=7) consisted of 200 ml Erlenmeyer flasks containing 100 ml of either untreated seawater (n=3) or autoclaved seawater (n=3). The three strains were inoculated into separate flasks to reach the final concentration of approximately 5×10^3 CFU/ml. One flask containing untreated seawater was not inoculated with any of the strains and served as a control to observe the behaviour of the indigenous microflora alone.

Flasks were maintained at room temperature under gentle agitation. Samples were collected from each flask immediately after inoculation (day 0) and after 8 h, 24 h (day 1), 48 h (day 2), 1 week (day 7), 2 weeks (day 14), 3 weeks (day 21), and 4 weeks (day 28). For each sampling time, counts of the strains under study were performed with the antibiotic-selective media described above. In addition, numbers of total culturable bacteria were enumerated on Tryptic Soya agar following 48 hours of incubation at 30°C.

2.5.3 *In situ* pond experiment

The *in situ* experiment was performed in a freshwater pond using membrane-filter chambers (Technical Services, Montana State University, USA). These

chambers are designed to allow diffusion of water and solutes but without diffusion of the bacteria contained in the chambers (Fig. 2.2). Two bacterial suspensions containing approximately 10^4 CFU/ml of each strain were prepared using water collected from the pond, either without any previous treatment (chamber 1) or autoclaved (sterilised) (chamber 2). Immediately after preparation, 100 ml of the suspensions were inoculated into the chambers, which were then immersed into the pond.

During the experimental period (November-December 2000), the water temperature in the daytime varied from 6 to 9°C, the pH was approximately 7.5 and the concentration of dissolved oxygen was between 11 and 12 mg/L. Samples were collected from the chambers and the pond immediately after inoculation (day 0) and after 4 days (day 4), 1 week (day 7), 2 weeks (day 14), 3 weeks (day 21), and 4 weeks (day 28). Enumeration of the strains under study and total culturable bacteria was performed as described previously. At day 28, representative colonies were isolated from the media used for enumeration of the strains and characterised by phenotypic tests (i.e. colony morphology, cell morphology, glucose O/F and cytochrome oxydase test) and ribotyping to confirm strain identity.

An enrichment procedure was used for detection of stressed cells following 28 days of incubation into the chambers. For this purpose, 1 ml of water was collected from each of the chambers and serial ten-fold dilutions were prepared using peptone buffered water. Dilution tubes were incubated for 48 h at 30°C under gentle agitation and, subsequently analysed for bacterial growth visually.

Figure 2.2. A membrane-filter chamber used in the *in situ* pond experiment.

3 Effects of hospital and pharmaceutical waste effluent on the prevalence of resistant *Acinetobacter* in the recipient sewers

Waste effluent from hospitals contains high numbers of resistant bacteria [48,58,59] and antibiotic residues at concentrations able to inhibit the growth of susceptible bacteria [35]. Accordingly, hospital waste effluent could increase the numbers of resistant bacteria in the recipient sewers by both mechanisms of introduction and selection for resistant bacteria. The effects caused by the introduction of hospital waste effluent into the sewage system were prior to our investigation. Furthermore, no comparison was made with the effects produced by waste effluent from other potential sources of resistant bacteria and antibiotic residues, like pharmaceutical plants producing or manufacturing antibiotics.

In the first part of the project, we investigated the effects of waste effluent from a hospital (section 3.1) and a pharmaceutical plant manufacturing antibiotic products (section 3.2). The levels of susceptibility to six antimicrobial agents were determined in a total of 385 *Acinetobacter* isolates obtained from sites situated upstream (site A) and downstream (sites B and C) from the discharge points of the hospital (n=180) and the pharmaceutical plant (n=205).

In addition, a subset of 43 *Acinetobacter* isolates was characterised by biochemical tests and plasmid profiles to detect possible changes in the composition of the bacterial population associated with the discharge of waste effluent from the pharmaceutical plant (section 3.2). The methods used for sampling, bacterial isolation, antimicrobial susceptibility testing, phenotypic characterisation and plasmid profiling are described in chapter 2.

3.1 Effects of hospital waste effluent

Only minor differences were observed in the levels of antibiotic resistance between *Acinetobacter* isolates from sites situated upstream and downstream from the hospital discharge point (Table 3.1). Oxytetracycline resistance was detected only at sites B and C, and represented 37.2% and 12.5% of the total isolates from those sites, respectively. The occurrence of oxytetracycline-resistant isolates was significantly higher at site B compared with site A (P<0.01). However, at site C, 500 m beyond the discharge point, the levels of oxytetracycline resistance were significantly reduced compared with site B (P<0.01).

Surprisingly, the occurrence of chloramphenicol-resistant isolates observed at site B was significantly higher than that observed at site A (P<0.01). In relation to the other four antibiotics used for susceptibility testing (ampicillin, ciprofloxacin, gentamicin and sulfametoxazole), no significant differences

were found between different sites in the occurrence of resistant isolates. Independent of the sampling site, most isolates were either susceptible to all antibiotics tested (51.7%) or resistant to only one compound (43.9%). Isolates resistant to more than two antibiotics were not detected at site A, and were rarely observed at sites B (1.3%) and C (4.7%) (Fig. 3.1).

Table 3.1. Percentages of antibiotic resistance in 180 *Acinetobacter* isolates from the sewers receiving waste effluent from a hospital.

Antibiotic	% of resistant isolates		
	Site A (n=38)	Site B (n=78)	Site C (n=74)
Amoxicillin	2.6	2.6	3.1
Ciprofloxacin	0	2.6	0
Chloramphenicol	55.3[a]	16.7	26.6
Gentamicin	0	0	3.1
Oxytetracycline	0	37.2 [b]	12.5 [c]
Sulfamethoxazole	0	2.6	4.7

[a] Statistically significant decrease in the numbers of resistant isolates occurring at site B compared with site A.
[a] Statistically significant increase in the numbers of resistant isolates occurring at site B compared with site A.
[c] Statistically significant decrease in the numbers of resistant isolates occurring at site C compared with site B.

Figure 3.1. Percentages of antibiotic sensitivity, resistance to one antibiotic and multiple resistance in 180 *Acinetobacter* isolates from the sewers receiving waste effluent from a hospital.
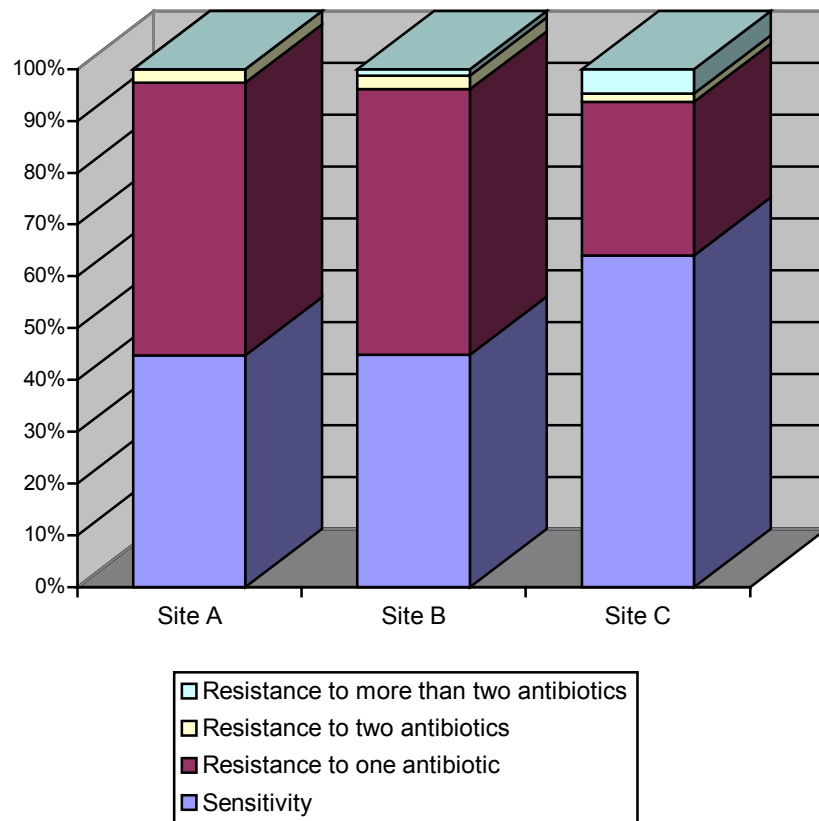
The reduction in the percentage of chloramphenicol resistance observed at downstream sites is difficult to explain. A possible explanation could be the presence of high numbers of chloramphenicol-sensitive strains in the hospital effluent, as this drug is no longer used in human medicine. Alternatively, substances present in the hospital effluent could affect the survival of chloramphenicol-resistant strains isolated upstream from the discharge point.

Indeed, among the the 21 chloramphenicol-resistant isolates obtained from site A, 20 isolates were susceptible to the other five antibiotics used for susceptibility testing, and only a single isolate was resistant to amoxicillin.

The total numbers of culturable bacteria were markedly higher at the two sites B and C (10^6 to 10^7 CFU/ml) compared to site A (10^3 CFU/ml), indicating that the hospital effluent introduced high numbers of bacteria into the recipient sewers. Since the prevalence of *Acinetobacter* among total culturable bacteria was higher at sites B (23%) and C (9%) than at site A (<1%), it could be deduced that this organism was widely distributed in the hospital effluent. Indeed, it has recently been demonstrated that *Acinetobacter* is the most prevalent bacterial taxon (15% to 56%) among tetracycline-resistant heterotrophic bacteria in hospital waste effluent [60]. These data confirm the suitability of our choice of *Acinetobacter* as a bacterial indicator for monitoring antibiotic resistance in hospital waste effluent.

The results of our study are in accordance with those of a previous study conducted at the same hospital by the former Danish Water Quality Institute (VKI) [61]. Although different methods and target bacterial populations were used for monitoring of antibiotic resistance, both studies indicate a significant increase in the occurrence of tetracycline resistant bacteria associated with the discharge of waste effluent from the hospital. It is difficult to explain these results based on the data on national consumption of antibiotics in hospital care, as tetracyclines account for only a minor component of the antibiotics used in Danish hospitals in recent years [62]. However, resistance to this antibiotic could also be co-selected by exposure to other substances, since tetracycline resistance genes are often located on plasmids together with other resistance genes.

3.2 Effects of pharmaceutical waste effluent

Acinetobacter isolates from the two sites situated downstream from the pharmaceutical plant demonstrated drastically increased levels of antibiotic resistance compared with isolates from the site situated upstream (Table 3.2). Significantly, higher percentages of *Acinetobacter* isolates resistant to amoxicillin, chloramphenicol, gentamicin, oxytetracycline and sulfamethoxazole were obtained from site B in comparison with site A (P<0.01). No statistically significant differences were detected between the two sites situated downstream from the sewage discharge point, indicating that the increase in the percentages of resistant isolates persisted at least 250 m from the discharge point.

Table 3.2. Percentages of antibiotic resistance in 205 *Acinetobacteer* isolates from the sewers receiving waste effluent from a pharmaceutical plant.

Antibiotic	Antibiotic resistant isolates (%)		
	Site A (n=76)	Site B (n=72)	Site C (n=57)
Amoxicillin	10.5	26.4 [a]	31.6
Ciprofloxacin	1.3	0	0
Chloramphenicol	25.0	51.4 [a]	61.4
Gentamicin	0	20.8 [a]	12.3
Oxytetracycline	5.3	48.6 [a]	61.4
Sulfamethoxazole	0	51.4 [a]	57.9

[a] Statistically significant increase in the numbers of resistant isolates occurring at site B compared with site A.

High numbers of multiple-resistant *Acinetobacter* isolates were found at the sites situated downstream from the pharmaceutical plant (Fig. 3.2). Multiple resistance to three or more antibiotics was frequently observed among isolates from sites B (36.1%) and C (38.6%), whereas, this was not seen with isolates from site A. A statistically significant increase in the percentage of multiple-resistant isolates was shown for site B in comparison with site A (P<0.001), but only at the first three sampling times.

Resistance to sulfamethoxazole and oxytetracycline was often found associated among isolates from sites B (47.2%) and C (54.4%). This resistance pattern was observed in association with chloramphenicol resistance both among isolates from sites B (34.7%) and C (29.8%). Multiple resistance to all antibiotics used, except ciprofloxacin, was observed in 15.3% and 17.5% of the isolates from sites B and C, respectively. None of the above mentioned multiple resistance patterns were detected among isolates from site A.

Figure 3.2. Percentages of antibiotic sensitivity, resistance to one antibiotic and multiple resistance in 205 *Acinetobacter* isolates from the sewers receiving waste effluent from a pharmaceutical plant.
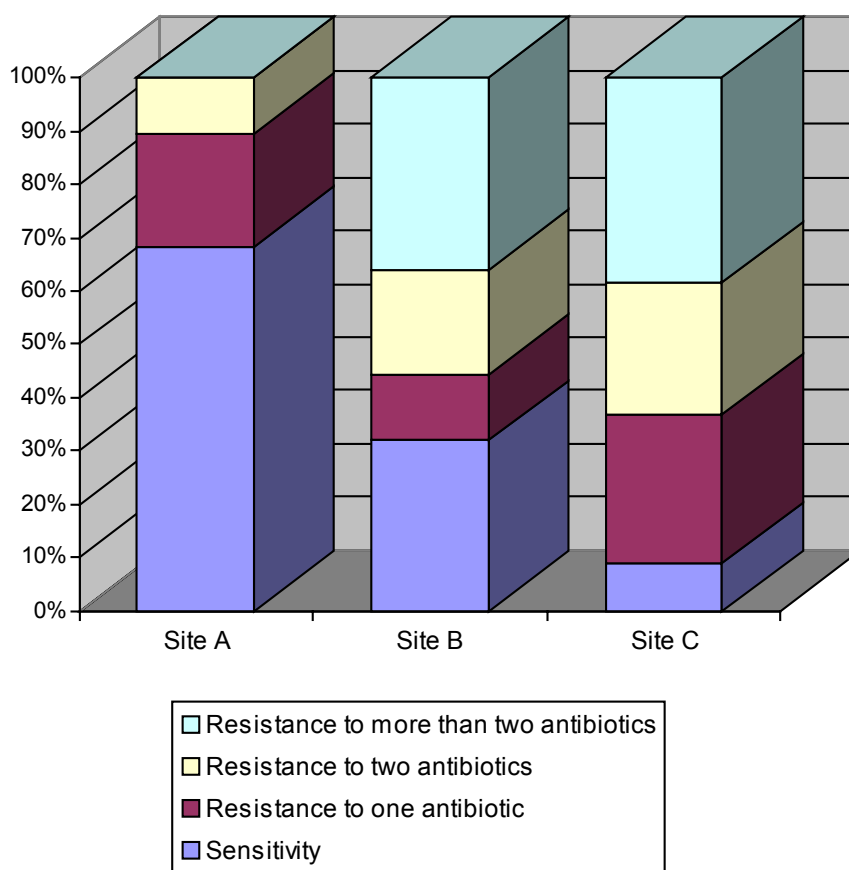
The observed increase in the prevalence of antibiotic resistance was associated with differences in the distribution of *Acinetobacter* species/strains between sites situated upstream and downstream from the pharmaceutical plant. *Acinetobacter* isolates from sites B and C showed different phenotypic patterns and plasmid profiles compared with isolates from site A. Seven different clones of *Acinetobacter* were found to be responsible for the high levels of multiple resistance observed at the two sites situated downstream from the pharmaceutical plant (Fig. 3.3).

According to phenotypic characterisation, the multiple-resistant strains isolated from the sewers receiving waste effluent from the pharmaceutical plant belonged to at least four different groups of *Acinetobacter* species: *A. lwoffii/A. johnsonii* (n=4), *Acb* complex (n=1), *A. junii* (n=1) and genomic species 16BJ (n=1). Some of the strains contained plasmids of similar size, but no single plasmid appeared to be present in all seven strains (Fig. 3.3). Consequently, the occurrence of multiple antibiotic resistance in the indigenous *Acinetobacter* population was not mediated by horizontal transfer of a unique plasmid structure.

Figure 3.3. Plasmid profiles of seven multiple-resistant *Acinetobacter* strains occurring in the sewers situated downstream from the pharmaceutical plant (lanes 1-7). Lanes R1-R2, reference strains *E. coli* 39R 861 and *E. coli* V517.
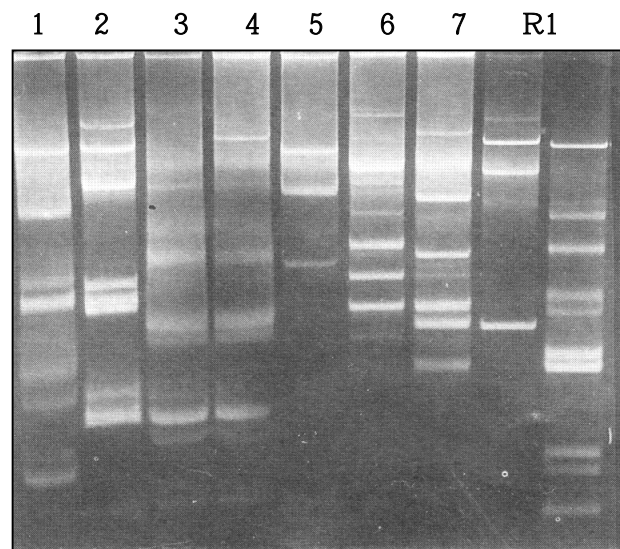
The combined use of antibiogram typing and plasmid profiling demonstrated that identical *Acinetobacter* strains were repeatedly isolated throughout the period of study. Isolates showing identical antibiotic resistance patterns, and identical or closely related plasmid profiles, were obtained from samples collected at different times or sites (Fig. 3.4). The clonal relationship of isolates showing identical plasmid profiles was further confirmed by Random Amplified Polymorphic DNA (RAPD) analysis, a DNA fingerprinting method for bacterial typing (data not shown). Based on these results, it could be concluded that multiple-resistant *Acinetobacter* clones, or presumptive clones, were established in the bacterial population of the sewers receiving waste effluent from the pharmaceutical plant.

Figure 3.4. Examples of identical or closely identical plasmid profiles in *Acinetobacter* isolates obtained at different times or from different sites situated downstream from the pharmaceutical plant (lanes 1-7). Lanes R1-R2, reference strains *E. coli* 39R 861 and *E. coli* V517.
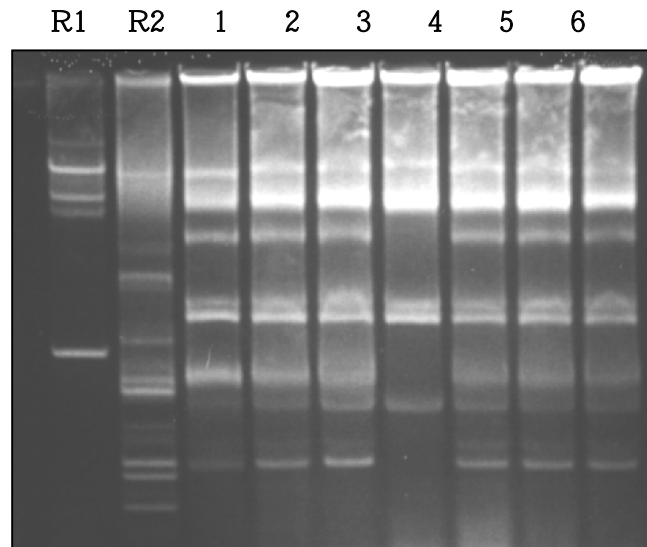
Previous studies on the effects of antibiotic resistance caused by the discharge of waste effluent from pharmaceutical plants, have been scarce. A brief report, previously conducted at the same pharmaceutical plant showed that the numbers of enterobacteria resistant to chloramphenicol, tetracyclines and aminoglycosides were higher at sites situated downstream from the effluent discharge point [63]. A similar effect on antibiotic resistance was observed in sewers receiving waste effluent from another pharmaceutical plant producing fusidic acid [63]. Therefore, according to the data currently available, it seems that waste effluent from pharmaceutical plants producing or manufacturing antibiotics enhances the occurrence of resistant bacteria in sewage.

The mechanisms by which waste effluent derived from antibiotic production or manufacture increases the numbers of resistant bacteria in sewage has not been investigated. At the pharmaceutical plant under study, products containing penicillins, tetracyclines, tylosin, and to a lesser extent aminoglycosides and sulfonamides, were manufactured during the period of sampling. At the end of each production cycle, the tanks where antibiotic agents had been produced were washed and the residual water, containing antibiotic residues and possibly resistant bacteria, was released directly into the sewage system. Consequently, the increased occurrence of resistant bacteria in the recipient sewers could be due to introduction of either antibiotic residues or resistant bacteria.

3.3 Conclusions

Waste effluent from the pharmaceutical plant was shown to have a higher impact on both single and multiple antibiotic resistance compared with waste effluent from the hospital, which had little effect on occurrence of resistant *Acinetobacter* in the recipient sewers. Therefore, waste effluent from pharmaceutical plants producing or manufacturing antibiotics could represent an important source for the occurrence of resistant bacteria in sewage.

This study indicates that human activities other than the indiscriminate use of antibiotics in human medicine, animal husbandry and agriculture, may disrupt the microbial balance in favour of resistant bacteria. Antibiotic resistance can develop not only in humans and in animals treated with antibiotics, but also in aquatic environments where antibiotics are present as residues derived from industrial production.

Further studies are necessary to assess the actual impact of antibiotic manufacturing on the spread of resistant bacteria in sewage. In particular, more pharmaceutical plants should be investigated, since various factors, such as type and size of production, could influence the occurrence of resistant bacteria and/or antibiotic residues in waste effluent derived from antibiotic production or manufacturing. Furthermore, studies should be implemented to investigate whether antibiotic residues originating from this source may negatively affect the biological treatment process at sewage treatment plants.

4 Effects of sewage treatment on total numbers and percentages of resistant bacteria

The possibility that resistant bacteria occurring in sewage can reach natural aquatic habitats is correlated to their ability to survive sewage treatment. It is generally assumed that sewage treatment determines a marked reduction in the bacterial numbers, including the total numbers of resistant bacteria. However, some studies have documented higher percentages of multiple-resistant bacteria in treated sewage compared with raw sewage [64,65], indicating that resistant and susceptible bacterial populations may not be equally affected by treatment.

In the second part of the project, we investigated the effects of tertiary sewage treatment on total numbers (section 4.1) and percentages (section 4.2) of resistant bacteria. The numbers of resistant bacteria in raw sewage, treated sewage and anaerobically digested sludge from two large-scale treatment plants (Avedøre Spillevandscenter I/S and Lynettefællesskabet I/S) were enumerated on media containing ampicillin, gentamicin, tetracycline or all three antibiotics (antibiotic selective method). Bacteriological counts were determined using media selective for two distinct bacterial populations, i.e. coliforms and acinetobacters. This afforded possible discrimination as to whether the effects of sewage treatment varied among different bacterial populations.

In addition, the levels of susceptibility to 14 antibiotics were determined in 442 *Acinetobacter* isolates obtained from culture on agar media without antibiotics (antibiotic non-selective method) and identified at the genus level. The use of two different methods for quantitative assessment of antibiotic resistance allowed us to compare results obtained by different methods. The description of the sampling sites and the methods used for sampling, bacteriological counts, bacterial isolation, identification, antibiotic susceptibility testing and statistical analysis are described in Chapter 2.

4.1 Effects on total numbers of resistant bacteria

At both plants under study, sewage treatment was associated with a marked reduction in the total numbers of resistant bacteria. The numbers of coliforms resistant to ampicillin, gentamicin and tetracycline were generally 100 to 1000 times lower in treated sewage compared with raw sewage (Figs. 4.1). The only exception was the first sampling time (August) at Lynetten plant, where the numbers of resistant coliforms in treated sewage were about 10 times lower compared with raw sewage. Similar results were seen with acinetobacters (see Paper 4).

Independent of the sample type, coliforms resistant to ampicillin occurred more frequently in comparison with bacteria resistant to gentamicin or tetracycline (Fig. 4.1). Bacteria resistant to all three antibiotics in raw sewage

were relatively frequent with regard to both coliforms (10^2 to 10^3 CFU/ml) and acinetobacters (10^3 to 10^4 CFU/ml). Such multiple-resistant bacteria were not detected in treated sewage from the Avedøre plant, and were seen only at low numbers (#10^2 CFU/ml) in treated sewage from Lynetten.

Figure 4.1. Numbers of coliforms resistant to ampicillin (AMP-resistant), gentamicin (GEN-resistant), tetracycline (TET-resistant) or all three antibiotics (Multi-resistant) in raw and treated sewage from the two treatment plants under study.
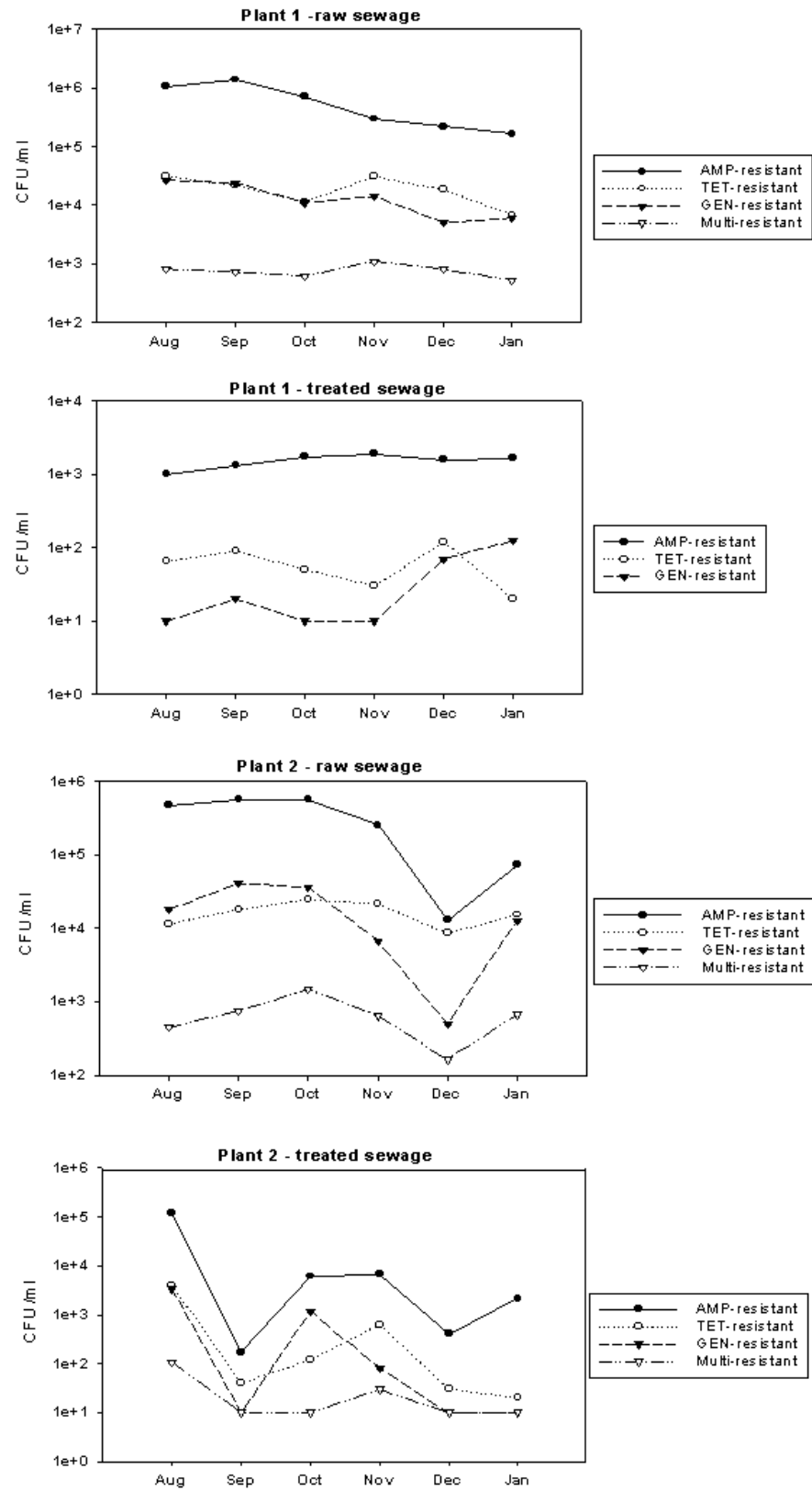
Single- and multiple-resistant bacteria occurred in high numbers in digested sludge from both plants (Table 4.1), indicating that anaerobic digestion had little effect on the total numbers of resistant bacteria occurring in sludge. Differences were observed between the two plants, with sludge from Avedøre plant containing lower numbers of resistant bacteria compared with sludge from Lynetten plant. Since the total numbers of resistant bacteria occurring in raw sewage were similar at the two plants (Fig. 4.1), it appeared that the process of sludge treatment at the Avedøre plant reduced the numbers of resistant bacteria more efficiently than at Lynetten plant. However, at both plants digested sludge was further treated by incineration (850°C for 2 sec), which eliminates any form of bacterial life. Furthermore, the resulting ash was not used for agricultural purposes. Therefore, these results do not imply any potential risks for the dissemination of resistant bacteria in the environment through use of sludge in agriculture.

Table 4.1. Average numbers (CFU/g) of coliforms and acinetobacters resistant to antibiotics occurring in anaerobically digested sludge.

Antibiotic	Avedøre plant		Lynetten plant	
	Coliforms	Acinetobacters	Coliforms	Acinetobacters
Ampicillin	4.4×10^4	9.3×10^4	9.1×10^5	1.0×10^6
Gentamicin	1.3×10^3	3.2×10^4	1.9×10^4	2.5×10^5
Tetracycline	5.3×10^3	2.2×10^4	4.5×10^4	1.5×10^6
All 3 antibiotics	5.7×10^2	1.3×10^3	2.0×10^3	7.9×10^3

The average percentages of acinetobacters and coliforms among total heterotrophic bacteria for each plant and sample type are shown in Table 4.2. Independent of the sample type, acinetobacters were more prevalent in the total heterotrophic bacterial population compared with coliforms. In the Lynetten plant, the prevalence of coliforms in treated sewage was significantly higher than in raw sewage (P<0.05). No other significant differences were observed in the prevalence of the two bacterial populations in different sample types (Table 4.2).

Table 4.2. Average percentages of coliforms and acinetobacters among total culturable bacteria in raw sewage, treated sewage and anaerobically digested sludge.

Bacterial population	Avedøre plant			Lynetten plant		
	Raw sewage	Treated sewage	Digested sludge	Raw sewage	Treated sewage	Digested sludge
Coliforms	12.6	14.8	8.0	5.8	15.1*	7.0
Acinetobacters	19.3	23.6	25.7	26.1	72.9	28.6

*The percentage of coliforms in treated sewage was significantly higher than in raw sewage (P<0.05).

4.2 Effects on percentages of resistant bacteria

The relative numbers of antibiotic-resistant coliforms and acinetobacters were not significantly increased by sewage treatment (Table 4.3). On the contrary, at the Avedøre plant the percentage of ampicillin-resistant acinetobacters in treated sewage was significantly lower than in raw sewage (P<0.05). At the same plant, digested sludge contained significantly lower percentages of ampicillin-resistant acinetobacters (P<0.005), ampicillin-resistant coliforms (P<0.05) and gentamicin-resistant coliforms (P<0.05) compared with raw sewage, indicating that anaerobic digestion also had a positive effect on percentages of resistant bacteria.

Although these results clearly indicate that the relative numbers of single- and multiple-resistant bacteria were not increased by sewage treatment, it should be noted that strains demonstrating multiple-resistant to ampicillin, gentamicin and tetracycline, such as those detected in treated sewage from the Lynetten plant, are unlikely to occur naturally in the environment. Indeed, the occurrence of such multiple-resistant strains was not detected in seawater collected from Øresund that was analysed using the same media and antibiotic concentrations employed in this study (data not shown).

The average percentages of resistant bacteria occurring in raw sewage, treated sewage and digested sludge are shown in Table 4.3. Ampicillin resistance occurred more frequently than tetracycline and gentamicin resistance in both coliforms and acinetobacters. This could be due to the fact that many bacterial species are intrinsically resistant to penicillins in general but particularly to ampicillin. Coliforms were generally more resistant to ampicillin and less resistant to gentamicin and tetracycline compared with acinetobacters (Table 4.3).

Table 4.3. Average percentages (%) of antibiotic-resistant coliforms and acinetobacters in raw sewage, treated sewage and digested sludge determined by counts on media countaining antibiotics.

| Antibiotic[a] | Avedøre plant | | | | | | Lynetten plant | | | | | |
| | coliforms | | | acinetobacters | | | coliforms | | | acinetobacters | | |
	Raw sewage	Treated Sewage	Digested Sludge	Raw sewage	Treated sewage	Digested sludge	Raw sewage	Treated sewage	Digested sludge	Raw sewage	Treated sewage	Digested sludge
Ampicillin	51.4	60.3	34.2[b]	47.2	27.0[a]	20.0[b]	47.7	50.5	38.5	18.2	22.3	12.2
Gentamicin	1.4	1.8	1.1[c]	3.9	7.2	5.5	3.3	3.1	1.7	3.4	5.8	3.8
Tetracycline	2.0	2.2	3.7	10.8	11.5	4.3	4.9	2.2	3.2	12.3	12.9	16.7
All antibiotics	0.1	-[c]	0.3	0.1	-[c]	0.3	0.2	0.3	0.3	0.3	0.3	0.1

[a] The percentage of resistant bacteria in treated sewage was significantly lower than in raw sewage (P<0.05).
[b] The percentage of resistant bacteria in digested sludge was significantly lower than in raw sewage (P<0.05).
[c] Multiple resistance to ampicillin, gentamicin and tetracycline was not detected in treated sewage from Avedøre.

The results obtained by bacteriological counts were confirmed by antibiotic susceptibility testing of *Acinetobacter* isolates. Among the 14 antibiotics tested, statistically significant differences were rarely observed in the percentages of resistant isolates originating from different sample types (Table 4.4), indicating a limited effect of sewage treatment on percentages of resistant bacteria. Only the percentage of isolates resistant to nalidixic acid was significantly higher in treated sewage than in raw sewage (P<0.05). Furthermore, the increase in the percentage of nalidixic acid resistance in treated sewage was observed only at the Avedøre plant, indicating that this effect was not generally associated with sewage treatment *per se*, but more specific to the conditions occurring at this particular plant.

The highest overall percentages of *Acinetobacter* isolates resistant to antibiotics were observed for aztreonam (38.0%), cefoxitin (23.8%), chloramphenicol (18.6%), cefotaxime (10.2%), tetracycline (8.4%) and nalidixic acid (6.8%). Resistances to amikacin, imipenem and tobramycin were not detected. For the remaining antibiotics, the percentages of resistant isolates were less than 3% (Table 4.4). The percentage of ampicillin resistance among *Acinetobacter* isolates was very low (0.9%) in comparison with the percentages of ampicillin-resistant acinetobacters obtained by bacteriological counts on Baumann agar containing this antibiotic (see Table 4.3). This is probably because bacterial

species other than *Acinetobacter*, including bacteria intrinsically resistant to penicillins (e.g. *Pseudomonas*), are able to grow on this medium [54].

As described in section 2.2.2, in some cases antibiotic resistance was defined according to two different breakpoints, a breakpoint determined empirically based on the distribution of the inhibition zone diameters and a breakpoint used in clinical practice for definition of antibiotic resistance in clinical *Acinetobacter* isolates. Although the two breakpoints differed only by 1 to 3 mm, such a difference was seen to substantially influence the overall percentages of resistance to aztreonam and chloramphenicol (Table 4.4).

For chloramphenicol and tetracycline, the determination of the breakpoint also influenced the results of the statistical analysis (Table 4.4). According to one breakpoint value, isolates resistant to chloramphenicol occurred more frequently in digested sludge when compared with raw sewage at both the Avedøre plant (P<0.005) and the Lynetten plant (P<0.05), and no statistically significant differences were observed in the occurrence of tetracycline-resistant isolates between different sample types. On the other hand, the use of the second breakpoint value showed that the differences in the occurrence of chloramphenicol-resistant isolates between digested sludge and raw sewage were no longer statistically significant and only tetracycline-resistant isolates from Lynetten occurred more frequently in digested sludge compared with raw sewage (P<0.01).

Table 4.4. Percentages (%) of antibiotic resistance among *Acinetobacter* isolates from raw sewage, treated sewage and digested sludge.

Antibiotic	Total (n=442)	Avedøre plant			Lynetten plant		
		raw sewage (n=65)	treated sewage (n=60)	digested sludge (n=83)	raw sewage (n=63)	treated sewage (n=102)	digested sludge (n=69)
Ampicillin	0.9	0	0	1.2	1.6	0	2.9
Amikacin	0	0	0	0	0	0	0
Aztreonam 1*	38.0	33.8	45.0	33.7	39.7	45.1	29.0
Aztreonam 2*	28.1	26.2	30.0	26.5	25.4	35.3	21.7
Cefotaxime	10.2	16.9	5.0[a]	4.8[b]	11.1	11.7	11.6
Cefoxitin	23.8	27.7	21.7	18.1	22.2	22.3	31.9
Chloramphenicol 1*	18.6	15.4	18.3	34.9[d]	7.9	12.7	20.3[d]
Chloramphenicol 2*	5.7	7.7	6.7	14.5	1.6	0	4.3
Ciprofloxacin	2.7	0	0	1.2	4.8	5.9	2.9
Gentamicin	0.7	0	0	1.2	1.6	0	1.4
Imipenem	0	0	0	0	0	0	0
Nalidixic acid	6.8	1.5	10.0[c]	3.6	11.1	5.9	10.1
Piperacillin	0.7	0	0	0	3.2	1.0	0
Sulf.+ trimethoprim	1.1	4.6	0	0	1.6	0	1.4
Tetracycline 1*	8.4	9.2	13.3	3.6	6.3	6.9	13.0
Tetracycline 2*	7.7	7.7	13.3	3.6	3.2	6.9	13.0[d]
Tobramycin	0	0	0	0	0	0	0

* Resistances to aztreonam, chloramphenicol and tetracycline were defined according to two different breakpoints.
[a] The percentage of resistant isolates in treated sewage was significantly lower than in raw sewage (P<0.05).
[b] The percentage of resistant isolates in digested sludge was significantly lower than in raw sewage (P<0.05).
[c] The percentage of resistant isolates in treated sewage was significantly higher than in raw sewage (P<0.05).
[d] The percentage of resistant isolates in digested sludge was significantly higher than in raw sewage (P<0.05).

The analysis of the distribution of the inhibition zone diameters showed an atypical distribution for aztreonam-resistance and chloramphenicol-resistance among *Acinetobacter* isolates, which made the distinction between resistant and susceptible populations quite arbitrary (Paper 4). This type of distribution indicates the occurrence of multiple and gradual levels of resistance to these compounds. A similar distribution was observed also for cefoxitin and cefotaxime, although changes in the selection of the breakpoint values for definition of resistance to these antibiotics did not significantly affect the results of the study.

It should be noted that *Acinetobacter* isolates obtained from the two plants showed markedly lower percentages of single and multiple antibiotic resistance in comparison with *Acinetobacter* isolates previously obtained from sewers receiving waste effluent from a pharmaceutical plant (see section 3.2). The fact that this pharmaceutical plant was located in the catchment area of the Avedøre plant suggests that the numbers of resistant bacteria were substantially reduced along the sewage system before sewage reached the treatment plant.

The results of our study are in contrast with some previous studies [64,65], which indicated an increase in the percentage of multiple-resistant bacteria following sewage treatment. The divergence could be due to either factors affecting the efficiency of removal of resistant bacteria at different plants (e.g. initial composition of sewage, type of treatment, plant operation, etc.) or differences in the bacterial indicators and methods used for quantitative assessment of antibiotic resistance (e.g. type of medium, definition of the breakpoint value, etc.). Indeed, even in this study, slightly different results were obtained depending on the plant, the target bacterial population and the antibiotic under study, as well as on the method and the breakpoint values used to define antibiotic resistance.

4.3   Conclusions

Based on the analysis of samples obtained from the two large-scale sewage treatment plants during a period of six months, it can be concluded that sewage treatment substantially reduces the total numbers of resistant bacteria without increasing their relative numbers. In some cases, the relative numbers of resistant bacteria in treated sewage appeared even to be reduced in comparison with raw sewage.

Nevertheless, it was shown that in some cases low numbers of multiple-resistant bacteria survived sewage treatment and persisted in treated sewage. Consequently, the release of municipal sewage effluents into natural aquatic habitats appears to contribute to the spread of multiple antibiotic resistance in the indigenous aquatic microflora. Similarly, anaerobically digested sludge could contribute to the dissemination of multiple-resistant bacteria when applied directly to agricultural land without any further treatment. In countries such as Denmark, where further treatment is required for the use of sewage sludge as a fertilizer, it would be relevant to investigate the effects of post-treatment and storage of digested sludge on the occurrence of multiple-resistant bacteria.

The results of this investigation indicate that there is a need to understand how long multiple-resistant bacteria originating from municipal sewage effluents are able to survive after they are introduced in natural aquatic

habitats. Furthermore, their ability to retain their resistance properties and transfer resistance genes to aquatic bacteria should also be elucidated. The results of our studies on survival and *in vitro* transfer of antibiotic resistance by multiple-resistant strains isolated from the municipal sewage effluent of the Lynetten plant are described in Chapter 5.

5 Spread of resistant bacteria and resistance genes by municipal sewage effluents

The results presented in Chapter 4 showed that, although in low numbers, multiple-resistant bacteria are able to survive sewage treatment and reach natural aquatic habitats through their presence in treated sewage. As some types of multiple-resistant bacteria are unlikely to occur naturally in the aquatic environment, a possible risk could be that novel resistance genes are taken up by the indigenous microflora and spread by mechanisms of horizontal gene transfer. The actual environmental impact incurred depends on the ability of such multiple-resistant bacteria to survive in the aquatic environment, retain their antibiotic resistance properties and transfer resistance genes to the indigenous microflora.

In the Part III of the project, we investigated the fate of multiple-resistant bacteria occurring in treated sewage, and more generally, the impact of municipal sewage effluents on the spread of antibiotic resistance. Multiple-resistant strains isolated from treated sewage were studied for their ability to survive and retain antibiotic resistance in natural waters (section 5.1). The transfer of tetracycline resistance genes from bacteria in sewage to bacteria in natural aquatic habitats was investigated by laboratory mating experiments (section 5.2). Finally, the impact of municipal sewage effluents on spread of antibiotic resistance was evaluated by comparing the occurrence of resistant bacteria in blue mussels exposed to treated sewage and blue mussels collected from unpolluted sites (section 5.3).

5.1 Survival in the environment of resistant bacteria originating from sewage

The survival in natural waters of three multiple-resistant strains isolated from the effluent of the Lynetten plant was studied by laboratory seawater microcosms (section 5.1.1) and membrane-filter chambers immersed in a freshwater pond (section 5.1.2). The strains represented three different bacterial species: *Acinetobacter johnsonii*, *Escherichia coli* and *Citrobacter freundii*. Survival experiments were performed both in the presence (i.e. untreated water) and in the absence (i.e. autoclaved water) of the indigenous microflora. A detailed description of the methods used is provided in Chapter 2 (section 2.5).

5.1.1 Survival in laboratory seawater microcosms

Growth of the multiple-resistant strains was observed during the 48 hours following the inoculation of the strains into the microcosms (Fig. 5.1). The initial growth of the multiple-resistant strains was likely to be due to the incubation conditions used to maintain the microcosms in the laboratory rather than to a particular ability of the strains to multiply in seawater *per se*,

since similar growth was also observed in the microcosm containing the indigenous microflora alone (Fig. 5.1).

After 48 hours of incubation, the numbers of multiple-resistant strains gradually declined (Fig. 5.1), whereas, the total numbers of culturable bacteria remained stable at approximately 10^5 CFU/ml in all microcosms (Fig. 5.1). All three multiple-resistant strains survived longer in autoclaved seawater (Fig. 5.1B) than in untreated seawater (Fig. 5.1A). A similar finding was seen in a previous study investigating the survival of another *E. coli* strain in seawater from Køge Bugt [66]. The reduced survival of *E. coli* in untreated seawater could be due to both antagonism of the indigenous microflora and predation by protozoa.

Figure 5.1. Survival of multiple-resistant strains isolated from treated sewage in laboratory seawater microcosms. The arrows indicate the detection limit (10 CFU/ml).
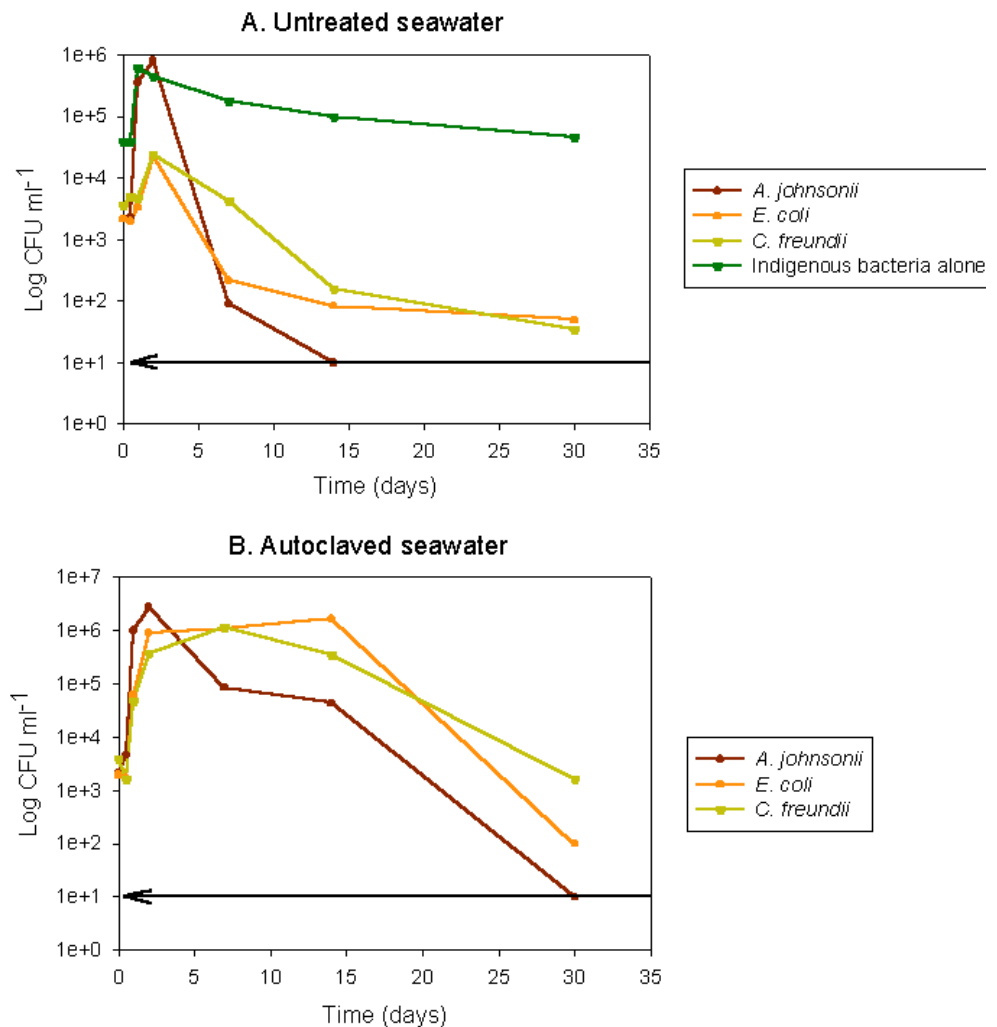
Surprisingly, the environmental species *A. johnsonii* showed a more rapid die-off compared with the enteric bacteria *E. coli* and *C. freundii*. In fact, the numbers of *A. johnsonii* strain fell below the detection limit (10 CFU/ml) after 14 days of incubation in untreated water, and after 30 days of incubation in autoclaved water, whereas, the *E. coli* and *C. freundii* strains were still detected after 30 days (Fig. 5.1).

The *E. coli* strain used in this study survived longer in seawater compared with a previously investigated laboratory strain of *E. coli* (i.e. *E. coli* K12), for which a survival of only five days was observed under similar laboratory conditions [66]. Physiological and/or structural changes associated with the exposure to various stressful conditions (e.g. antibiotic selective pressure, survival in sewage, survival of sewage treatment, etc.) could enable multiple-resistant bacteria in sewage to survive environmental stresses compared with the laboratory strains generally used in this kind of experiments.

This study demonstrated that multiple-resistant bacteria occurring in municipal sewage effluents were able to survive in seawater for at least one month following their inoculation into the microcosms. This result is particularly interesting in consideration of the fact that a low bacterial inoclula was used in comparison with previous studies concerning bacterial survival. The temperature in the laboratory microcosms (26°C to 30°C during the day) was higher compared with natural conditions. However, this should not detract from the validity of the result, as previous studies have demonstrated that *E. coli* survive longer in seawater at low temperatures [66].

5.1.2 Survival in a freshwater pond

The multiple-resistant strains showed a more rapid die-off in membrane-chambers immersed in a freshwater pond compared with laboratory seawater microcosms. In the chamber containing untreated pond water (Fig. 5.2A), the numbers of the multiple-resistant strains fell below the detection limit (1 CFU/ml) after either 21 days (*A. johnsonii* and *E. coli* strains) or 28 days (*C. freundii* strain). In the chamber containing autoclaved pond water (Fig. 5.2B), *E. coli* and *C. freundii* strains survived slightly longer compared with the *A. johnsonii* strain. This was also the case in the chamber containing untreated pond water. Only the *C. freundii* strain was recovered after 28 days, although at very low numbers (2 CFU/ml). The numbers of total bacteria were constant in the chamber containing untreated pond water, as well as outside of the chambers.

As for the previous experiment conducted in laboratory seawater microcosms (section 5.1), the *A. johnsonii* strain survived for a shorter period compared with the other two multiple-resistant strains under study. The strain could not be recovered after 28 days of incubation in the pond, even when an enrichment procedure in peptone buffered water was used for detection of damaged and stressed cells. Therefore, it appeared that the *A. johnsonii* strain was no longer present in the chambers.

The use of the enrichment procedure in peptone buffered water revealed that the *E. coli* strain and *C. freundii* strains were both present in the two chambers after 28 days. Bacterial isolates (n=15) obtained following inoculation of the enrichment culture on the selective medium for these two strains (i.e. MacConkey agar with added antibiotics) showed the same colony morphology, resistance pattern, plasmid profile and ribotype of the respective *C. freundii* strain (n=14) and *E. coli* strain (n=1). These results confirm that the multiple-resistant isolates obtained after 28 days were identical to the test strains initially inoculated into the chambers.

After 28 days of incubation in the pond, the *E. coli* strain and *C. freundii* strains could be recovered from the first two enrichment dilutions (10^{-1} and 10^{-2}), but not from further dilutions, indicating that the level of the two strains in

the chambers was between 10^2 and 10^3 CFU/ml. A proportion of the two strain populations were probably in a stressed state since lower bacterial densities were detected by direct plating on the selective media (Fig. 5.2).

Characterisation of the bacterial isolates obtained from the chambers after 28 days of incubation in the pond revealed that the strains generally maintained their original plasmid profiles and multiple resistance properties. Only three isolates showed slight variation in the number of plasmid bands compared with the strain originally inoculated into the chambers. Rare differences were observed with regard to the level of susceptibility to one antibiotic (i.e. cefoxitin), with two isolates showing larger inhibition zone diameters (32 mm) compared with the strain originally inoculated into the chamber (10 mm).

Figure 5.2. Survival of multiple-resistant strains isolated from treated sewage in membrane-filter chambers immersed in a pond.
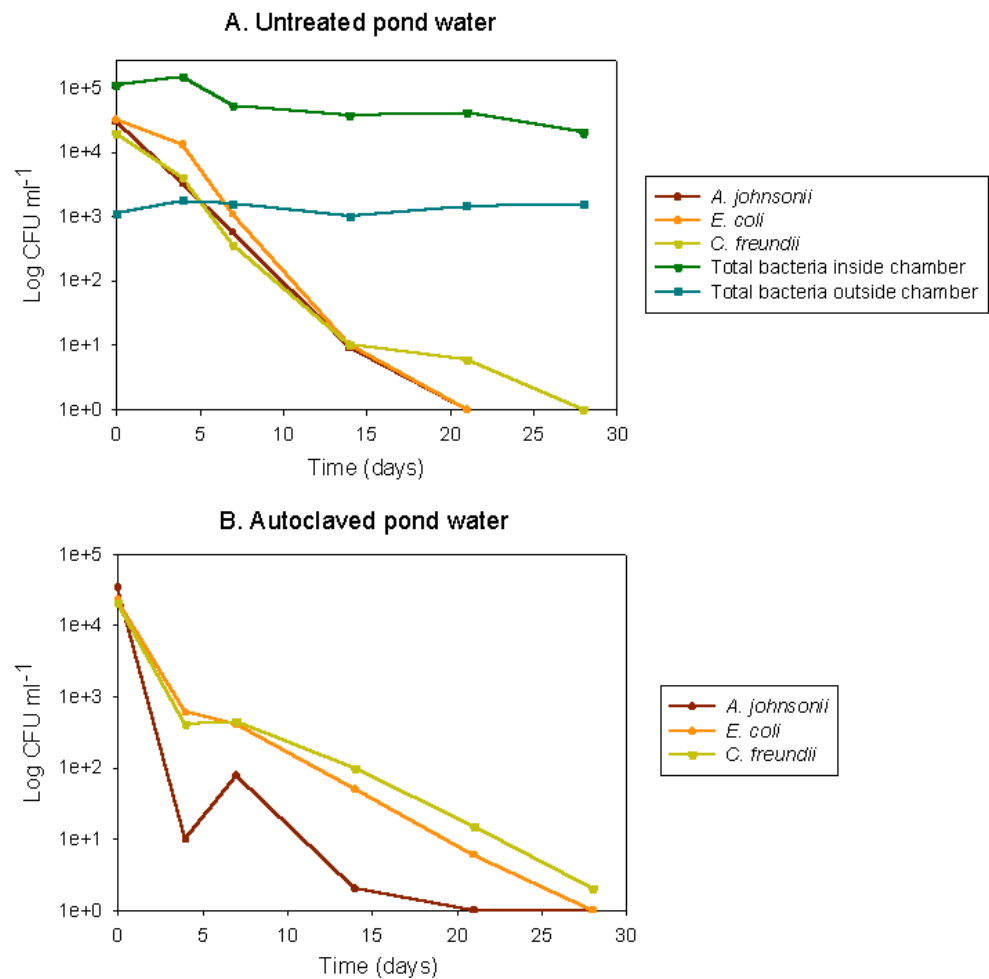
No bacteria showing the same multiple resistance patterns of the test strains were recovered in the pond. Furthermore, bacteria isolated randomly on MacConkey agar without antibiotics showed phenotypic and genotypic traits different from those of the test strains, indicating that the reduction in the numbers of the multiple-resistant strains observed during the experiment was actually due to bacterial die-off and not to loss of their multiple resistance properties.

The results of this experiment showed that two of the three multiple-resistant strains under study were able to survive in the freshwater pond for at least 28 days. Furthermore, the two strains maintained their multiple resistance

properties following one month of incubation under natural conditions. Therefore, it appears that multiple-resistant bacteria occurring in municipal sewage effluents can survive in natural freshwater environments for relatively long periods.

5.2 Transfer of resistance genes from sewage to aquatic bacteria

The possibility that tetracycline resistance is transferred from bacteria in sewage to bacteria in natural aquatic environments was studied under laboratory conditions. Mating experiments were carried out using unrelated tetracycline-resistant *Acinetobacter* strains isolated from sewage (n=10), aquacultural habitats (n=5) and clinical specimens (n=5) as donors and a tetracycline-sensitive *Acinetobacter* strain isolated from an unpolluted stream as a recipient (section 5.2.1). Furthermore, tetracycline-resistant *Acinetobacter* isolates from sewage (n=10), fish farms (n=5) and clinical specimens (n=35) were analysed by PCR for the occurrence of tetracycline resistance genes of the classes Tet A to E, with the aim to determine whether the same genes occur in *Acinetobacter* populations inhabiting different environments (section 5.2.2).

5.2.1 Laboratory mating experiments

Among the 20 *Acinetobacter* strains tested as potential donors of tetracycline resistance, transfer was demonstrated from only three aquatic strains, two from sewage and one from an aquaculture habitat (Paper 3). The two sewage strains capable of transferring tetracycline resistance originated from sewers receiving waste effluent from a hospital (strain LUH 5618) and a pharmaceutical plant (strain LUH 5613) (see Chapter 3). Transfer of tetracycline resistance was not apparent from any of the clinical *A. baumannii* strains to the aquatic recipient strain used in the laboratory matings.

Transfer did not occur when DNA from the donor strains was added to the recipient cultures and was not affected by the presence of deoxyribonuclease I, suggesting a conjugative nature of the transfer. Multiple plasmids of a relatively small size (<36 kb) were transferred from the donor strain LUH 5613 into the recipient strain (Paper 3). In the case of the donor strain LUH 5618, the transfer of tetracycline resistance was apparently not mediated by plasmids, since novel bands were not observed in the plasmid profile of the recipient strain (Paper 3).

This laboratory experiment showed that transfer of tetracycline resistance from sewage bacteria to bacteria living in natural aquatic habitats is possible. However, the limited number of strains used in the mating experiments does not permit broad conclusions on the frequency of such a transfer occurring in nature. Additionally, transfer did not occur between distantly related *Acinetobacter* species, suggesting the existence of physical or physiological barriers limiting the exchange of antibiotic resistance genes between different bacterial species belonging to the same genus.

5.2.2 Distribution of tetracycline resistance genes

Among the 15 aquatic *Acinetobacter* strains tested, three strains contained Tet B (Paper 3). The remaining aquatic strains contained unspecified tetracycline resistance determinants, which did not belong to any of the common classes

occurring in Gram-negative bacteria (Tet A, B, C, D, E, G and M). The three strains containing Tet B had previously been isolated from sewers receiving waste effluent from a pharmaceutical plant (see Chapter 3). The three strains belonged to different species according to both phenotypic and genotypic identification, indicating that Tet B was widespread in the *Acinetobacter* population of this habitat.

A different distribution of tetracycline resistance determinants was observed in clinical strains in comparison with the aquatic strains. Among the 35 clinical strains tested, 33 strains contained either Tet A (n=16) or Tet B (n=17) (Paper 3), indicating that these two classes of tetracycline resistance genes are widely distributed in *Acinetobacter* populations of hospital environments. The different distribution of tetracycline resistance genes in clinical and aquatic strains indirectly provides evidence that the predominant genes occurring in environmental *Acinetobacter* populations do not originate from clinical environments.

5.3 Occurrence of resistant bacteria in blue mussels exposed to treated sewage

The occurrence of resistant bacteria was studied in blue mussels collected from sites exposed to treated sewage (i.e. the outlets of the Avedøre and Lynetten plants) and a control site not exposed to treated sewage (i.e. Limnfjorden). Numbers of resistant bacteria were determined for both total culturable bacteria and *E. coli* as previously described (sections 2.2.5 and 2.2.6). The numbers of resistant bacteria in blue mussels either exposed or not exposed to treated sewage were compared to detect possible associations between antibiotic resistance and exposure to treated sewage.

5.3.1 Antibiotic resistance of total culturable bacteria in blue mussels

Occurrence of ampicillin resistance and to a lesser extent nalidixic acid resistance was more frequent than gentamicin and tetracycline resistance in the bacterial flora of mussels exposed to treated sewage (Table 5.1). A different distribution of antibiotic resistance was observed in the bacterial flora of mussels collected from Limfjorden, with nalidixic acid-resistant bacteria being surprisingly more frequent compared with ampicillin-resistant bacteria. Although at very low percentages (#0.1%), multiple-resistant bacteria occurred at the outlets of the two treatment plants. Multiple-resistant bacteria were also isolated from mussels collected at 100 m from the outlet of the Lynetten plant, but not from mussels collected at 100 m from the outlet of the Avedøre plant. This suggests that the presence of multiple-resistant bacteria in mussels living in the proximity of municipal sewage effluents depends on the specific conditions occurring at each treatment plant.

Higher percentages of ampicillin resistance were found in mussels exposed to treated sewage (12.9 to 95.5%) in comparison with mussels not exposed to treated sewage (1.5 to 5.4%). However, interpretation of the data on ampicillin resistance was difficult due to extremely variable percentages of resistant bacteria found in mussels collected from the same sites at different times (Table 5.2). The percentages of gentamicin and tetracycline resistance were low (<3%) independent of the origin of the mussels and the time of sampling.

Table 5.1. Counts of total and resistant bacteria (CFU/g) in blue mussels collected from sites exposed (Avedøre and Lynetten outlets) or not exposed (Lymfjorden) to treated sewage.

Source	Month	Total bacteria	Resistant bacteria					
			AMP	GEN	NAL	TET	AGT	AGNT
Avedøre 1	Aug	7.2×10^4	2.1×10^3	5.1×10^2	7.4×10^3	9.8×10^2	2.4×10^2	1.2×10^2
	Nov	2.2×10^6	2.1×10^6	1.4×10^4	1.6×10^5	9.3×10^3	1.4×10^3	1.0×10^3
Avedøre 2	Aug	4.3×10^4	1.2×10^4	N.D.	1.2×10^3	N.D.	N.D.	N.D.
	Nov	2.4×10^6	1.7×10^6	2.0×10^1	1.7×10^5	N.D.	N.D.	N.D.
Lynetten 1	Aug	5.0×10^6	2.5×10^6	4.6×10^4	1.2×10^6	1.4×10^5	1.7×10^3	2.2×10^3
	Nov	2.9×10^4	1.1×10^4	6.0×10^1	1.5×10^3	2.1×10^2	6.0×10^1	4.0×10^1
Lynetten 2	Aug	6.2×10^4	8.0×10^3	1.6×10^2	5.4×10^3	1.0×10^3	6.0×10^1	4.0×10^1
	Nov	2.2×10^6	2.0×10^6	1.8×10^4	3.4×10^5	7.9×10^3	9.2×10^2	1.8×10^3
Limfjorden	Aug	1.8×10^4	9.8×10^2	1.0×10^1	4.7×10^3	N.D.	N.D.	N.D.
	Nov	1.8×10^5	2.7×10^3	3.9×10^2	2.1×10^4	1.0×10^2	N.D.	N.D.

A, ampicillin; G, gentamicin; N, nalidixic acid; T, tetracycline; AGT, ampicillin, getamicin and tetracycline; AGNT, ampicillin, getamicin, nalidixic acid and tetracycline; Avedøre 1, outlet; Avedøre 2, ca. 100 m from the outlet; Lynetten 1, outlet; Lynetten 2, ca. 100 m from the outlet; N.D., not detected.

At sites exposed to treated sewage, a correlation was generally found between the percentages of antibiotic resistance and the distance from the outlets, with higher percentages of resistant bacteria observed in mussels collected from the outlets compared with mussels collected 100 m from the outlets (Table 5.2.). The only exception was the November sampling at the Lynetten plant where an opposite trend was observed (Table 5.2). This was probably because of the presence of strong currents in the direction of the sampling site situated 100 m from the outlet.

Table 5.2. Percentages (%) of resistant bacteria in blue mussels collected from sites exposed (Avedøre and Lynetten plants) or not exposed (Lymfjorden) to treated sewage.

Source	Month	Resistant bacteria					
		AMP	GEN	NAL	TET	AGT	AGNT
Avedøre 1	Aug	29.2	0.7	10.3	1.3	0.3	0.2
	Nov	95.5	0.6	7.3	0.4	0.1	<0.1
Avedøre 2	Aug	27.9	N.D.	2.8	N.D.	N.D.	N.D.
	Nov	70.8	<0.1	7.1	N.D.	N.D.	N.D.
Lynetten 1	Aug	50.0	0.9	24.0	2.8	<0.1	<0.1
	Nov	37.9	0.2	5.2	0.7	0.2	0.1
Lynetten 2	Aug	12.9	0.2	8.7	1.6	0.1%	0.1
	Nov	90.9	0.8	15.5	0.3	<0.1%	0.1
Limfjorden	Aug	5.4	0.1	26.1	N.D.	N.D.	N.D.
	Nov	1.5	0.2	11.6	0.1	N.D.	N.D.

A, ampicillin; G, gentamicin; N, nalidixic acid; T, tetracycline; AGT, ampicillin, getamicin and tetracycline; AGNT, ampicillin, getamicin, nalidixic acid and tetracycline; Avedøre 1, outlet; Avedøre 2, ca. 100 m from the outlet; Lynetten 1, outlet; Lynetten 2, ca. 100 m from the outlet; N.D., not detected.

This study demonstrates that, while resistance to single antibiotics can be found in any environment, including habitats characterised by low levels of pollutants, multiple-resistance to three or four different classes of antibiotics is not likely to occur in natural aquatic bacterial populations. Bacteria demonstrating multiple-resistance to ampicillin, gentamicin and tetracycline were found only in blue mussels exposed to treated sewage, confirming that municipal sewage effluents are likely to represent an important source for the dissemination of these bacteria in the environment.

5.3.2 Antibiotic resistance of *E. coli* in blue mussels

E. coli was only detected in blue mussels exposed to treated sewage, although only sporadically (Table 5.3). This situation did not allow comparison of data on antibiotic resistance between different sampling sites and times. Therefore, *E. coli* could not be used as a bacterial indicator for monitoring antibiotic resistance in blue mussels.

The selective medium used for enumeration of *E. coli* (i.e. TBX agar) could be usefully employed for microbiological analysis of blue mussels. Among 43 representative bacterial isolates tested by the API 20E identification system, 21 isolates (48.8%) were identified as *E. coli* with either a very good or good identification score, 14 isolates (32.6%) were identified as *E. coli* with a low discrimination profile, and 8 isolates (18.6%) showed a doubtful or unacceptable profile. Accordingly, the proportion of verified *E. coli* on the medium varied from 50% to 82% depending on the interpretation of the identification scores obtained by the API 20E identification system.

Table 5.3. Counts of total and resistant *E. coli* (CFU/g) in blue mussels collected from sites exposed (Avedøre and Lynetten plants) or not exposed (Lymfjorden) to treated sewage.

Source	Month	Total *E. coli*	Resistant *E. coli*					
			AMP	GEN	NAL	TET	AGT	AGNT
Avedøre 1	Aug	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	Nov	2.1×10^3	3.2×10^2 (15.2%)	2.0×10^1 (0.9%)	6.0×10^1 (2.9%)	1.2×10^2 (5.7%)	2.0×10^1 (0.9%)	N.D.
Avedøre 2	Aug	2.0×10^1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	Nov	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Lynetten 1	Aug	5.0×10^4	1.2×10^4 (24.0%)	1.9×10^2 (0.4%)	9.8×10^2 (2.0%)	4.8×10^3 (9.6%)	1.7×10^2 (0.3%)	8.0×10^1 (0.2%)
	Nov	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Lynetten 2	Aug	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	Nov	8.5×10^2	1.0×10^2 (11.8%)	N.D.	N.D.	4.0×10^1 (4.7%)	N.D.	N.D.
Limfjorden	Aug	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	Nov	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

A, ampicillin; G, gentamicin; N, nalidixic acid; T, tetracycline; AGT, ampicillin, getamicin and tetracycline; AGNT, ampicillin, getamicin, nalidixic acid and tetracycline; Avedøre 1, outlet; Avedøre 2, ca. 100 m from the outlet; Lynetten 1, outlet; Lynetten 2, ca. 100 m from the outlet; N.D., not detected.

5.4   Conclusions

The results of the survival experiments demonstrate that multiple-resistant strains isolated from treated sewage survived and retained antibiotic resistance for the duration of the study (28 days) in both seawater and freshwater. Above all, the multiple resistance properties of the strains under study remained unchanged after one month of incubation under natural conditions. Therefore, it seems that multiple-resistant bacteria occurring in municipal sewage effluents have sufficient time to transfer resistance genes to indigenous aquatic bacteria once they are released into natural aquatic environments.

Indeed, transfer of tetracycline resistance was shown to occurr under laboratory conditions from *Acinetobacter* strains isolated from sewage to a recipient strain originating from an unpolluted freshwater habitat. However, the actual ability of strains originating from sewage to transfer antibiotic resistance genes to aquatic bacteria under natural conditions needs further evaluation through laboratory experiments using larger numbers of recipient and donor strains resistant to different antibiotics, as well as *in situ* experiments. The lack of transfer of tetracycline resistance from clinical to aquatic *Acinetobacter* strains and the differences observed in the distribution of tetracycline resistance genes between the two bacterial populations, suggest that most tetracycline-resistant bacteria occurring in sewage and aquacultural habitats do not originate from clinical environments.

Bacteria that were multiple-resistant to ampicillin, gentamicin and tetracycline were found in treated sewage and in blue mussels collected at the outlets of municipal sewage effluents, but not in seawater, pond water or blue mussels collected from sites not exposed to treated sewage. This finding substantiates the hypothesis that municipal sewage effluents contribute to the dissemination of multiple-resistant bacteria in the environment. Consequently, future studies investigating the impact of munipal sewage effluents on the spread of

antibiotic resistance should focus on the occurrence of multiple-resistant bacteria rather than on the occurrence of bacteria resistant to single antibiotic compounds.

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Annex 1. Scientific papers and dissemination of results

The results achieved during the three years of this project have been disseminated by publications in international and national scientific journals, as well as by presentations at internationational conferences and national meetings. In all cases, the financial support of the Danish Environmental Protection Agency to the project was acknowleged.

The following is a list of publications in international peer-reviewed journals, which has been referred to throughout the report using the paper number designation:

Paper 1.   Guardabassi, L., Petersen, A., Olsen, J. E., Dalsgaard, A. (1998). Antibiotic resistance in *Acinetobacter* spp. isolated from sewers receiving waste effluent from a hospital and a pharmaceutical plant. *Applied and Environmental Microbiology*, 64:3499-3502.

Paper 2.   Guardabassi, L., Dalsgaard, A., Olsen, J. E. (1999). Phenotypic characterisation of *Acinetobacter* spp. isolated from aquatic sources. *Journal of Applied Microbiology*, 87:659-667.

Paper 3.   Guardabassi, L., Dijkshoorn, L., Olsen, J. E., Dalsgaard, A. (2000). Distribution and *in vitro* transfer of tetracycline resistance determinants in clinical and aquatic *Acinetobacter* strains. *Journal of Medical Microbiology*, 49: 929-936.

Paper 4.   Guardabassi, L., Lo Fo Wong, D. M. A., Dalsgaard, D. (2001). The effects of tertiary wastewater treatment on the prevalence of antimicrobial resistant bacteria. *Water Research*, 36: 1955-1964.

This is a list of presentations at conferences or meetings and publications in national journals:

1.   Guardabassi, L., Olsen, J. E., and Dalsgaard, A. (1998) The effect on the prevalence of antibiotic resistance of *Acinetobacter* spp. in sewage determined by the discharge of waste effluent from a hospital and a pharmaceutical plant. Poster presentation at the Symposium on Microbial Ecology, Halifax, Canada, 1998.

2.   Guardabassi, L. (1998) Sources of antibiotic resistance in the aquatic environment. Oral presentation at the Meeting of the Danish Association of Engineers, Copenhagen, Denmark.

3.   Guardabassi, L., Olsen, J. E., and Dalsgaard, A. (1999) Transfer of tetracycline resistance among *Acinetobacter* strains isolated from different habitats. Oral presentation at the 6th Symposium on Bacterial Genetics and Ecology, Florence, Italy.

4. Guardabassi, L. (2000) The use of *Acinetobacter* spp. as bacterial indicators for monitoring antimicrobial resistance in aquatic environments. Ph.D. thesis.

5. Guardabassi, L., and Dalsgaard, A. (2000). Wastewater treatment plants are unlikely to select for antimicrobial resistant bacteria. Poster presentation at the 1st World Congress of the International Water Association (IWA), Paris, France.

6. Guardabassi, L., Olsen, J. E., and Dalsgaard, A. (2000) The use of *Acinetobacter* spp. as bacterial indicators of antimicrobial resistance in aquatic environments. Poster presentation at the 5th Symposium on the Biology of *Acinetobacter*, Noordwijkerhout, The Netherlands.

7. Petersen, A., Guardabassi, L., Olsen, J. E., and Dalsgaard, A. (2000) Selektion of antibiotikaresistens i *Acinetobacter* spp. i spildevand. Dansk Veterinær Tidsskrift, 7: 6-9.

8. Dalsgaard, A., and Guardabassi, L. (2001) Antibiotika resistens i renseanlæg. Dansk Afløbs- og Spildevandsforening, 9: 7-8.

The listed papers can be found at the Danish National Library of Science and Medicine (http://www.dnlb.dk).