Antibiotic Resistance in Wastewater
Methicillin-resistant Staphylococcus aureus (MRSA)
and antibiotic resistance genes

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- Methicillin-resistant Staphylococcus aureus (MRSA) and antibiotic resistance genes

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Cover art: Trickling filters at Ryaverket, Göteborg, Sweden. Photo by: Stefan Börjesson.

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“Don't make fun of graduate students. They just made a terrible life choice”

- Marge Simpson (The Simpsons)
Abstract
A large part of the antibiotics consumed ends up in wastewater, and in the wastewater the antibiotics may exert selective pressure for or maintain resistance among microorganisms. Antibiotic resistant bacteria and genes encoding antibiotic resistance are commonly detected in wastewater, often at higher rates and concentrations compared to surface water. Wastewater can also provide favourable conditions for the growth of a diverse bacterial community, which constitutes a basis for the selection and spread of antibiotic resistance. Therefore, wastewater treatment plants have been suggested to play a role in the dissemination and development of antibiotic resistant bacteria. Methicillin-resistant Staphylococcus aureus (MRSA) is a large problem worldwide as a nosocomial pathogen, but knowledge is limited about occurrence in non-clinical environments, such as wastewater, and what role wastewater plays in dissemination and development of MRSA.

In this thesis we investigated the occurrence of MRSA in a full-scale wastewater treatment plant (WWTP). We also investigated the concentration of genes encoding resistance to aminoglycosides (aac(6')-Ie+aph(2'')), β-lactam antibiotics (mecA) and tetracyclines (tetA and tetB) in three wastewater-associated environments: (1) soil from an overland flow area treating landfill leachates, (2) biofilm from a municipal wastewater treatment plant, and (3) sludge from a hospital wastewater pipeline. In addition, concentrations of mecA, tetA and tetB were investigated over the treatment process in the WWTP. These investigations were performed to determine how the prevalence and concentration of MRSA and the antibiotic resistance genes are affected in wastewater and wastewater treatment processes over time. The occurrence of MRSA was investigated by cultivation and a commercially available real-time PCR assay. In order to determine concentrations of the genes aac(6')-Ie+aph(2''), mecA, tetA and tetB in wastewater we developed a LUX™ real-time PCR assay for each gene.

Using cultivation and real-time PCR we could for the first time describe the occurrence of MRSA in wastewater and show that it had a stable occurrence over time in a WWTP. MRSA could mainly be detected in the early treatment steps in the WWTP, and the wastewater treatment process reduced the number and diversity of cultivated MRSA. However, our results also indicate that the treatment process selects for strains with more extensive resistance and possibly higher virulence. The isolated wastewater MRSA strains were shown to have a close genetic relationship to clinical isolates, and no specific wastewater lineages
could be detected, indicating that they are a reflection of carriage in the community. Taken together, these data indicate that wastewater may be a potential reservoir for MRSA and that MRSA are more prevalent in wastewater than was previously thought.

The real-time PCR assays, for \textit{aac(6')-Ie+aph(2'')}, \textit{mecA}, \textit{tetA}, and \textit{tetB} that we developed, were shown to be sensitive, fast, and reproducible methods for detection and quantification of these genes in wastewater environments. The highest concentrations of all genes were observed in the hospital pipeline, and the lowest in the overland flow system, with \textit{tetA} and \textit{aac(6')-Ie+aph(2'')} detected in all three environments. In the full-scale WWTP, we continuously detected \textit{mecA}, \textit{tetA} and \textit{tetB} over the treatment process and over time. In addition, it was shown that the treatment process reduces concentrations of all three genes. The data presented in this thesis also indicate that the reduction for all three genes may be connected to the removal of biomass, and in the reduction of \textit{tetA} and \textit{tetB}, sedimentation and precipitation appear to play an important role.
Populärvetenskaplig sammanfattning

Resistenta gula stafylokocker (MRSA) och antibiotikaresistensgener förekommer i svenskt kommunalt avloppsvatten


I denna avhandling har vi försökt öka kunskapen om förekomst av MRSA i ickekliniska miljöer. Syftet var att undersöka om MRSA kan förekomma i avloppsvatten och hur MRSA och förekomsten av genen *mecA* (som ger methicillinresistens hos stafylokocker) påverkas av reningsprocesserna i ett kommunalt avloppsvattenreningsverk. Dessutom ville vi bestämma släktsskap mellan MRSA i avloppsvatten från reningsprocessen. MRSA i Sverige är fortfarande ovanligt men trenden är här som i övriga världen en ökning i antalet MRSA infektioner. Hur spridning och utveckling av MRSA sker i sjukvården är utförligt studerat, men vilken roll andra miljöer har för spridning och utveckling av MRSA är mindre känt.

I denna avhandling har vi försökt öka kunskapen om förekomst av MRSA i ickekliniska miljöer. Syftet var att undersöka om MRSA kan förekomma i avloppsvatten och hur MRSA och förekomsten av genen *mecA* (som ger methicillinresistens hos stafylokocker) påverkas av reningsprocesserna i ett kommunalt avloppsvattenreningsverk. Dessutom ville vi bestämma släktsskap mellan MRSA i avloppsvatten och MRSA inom sjukvården. För att kunna utföra projektet samlades vattenprover in från ett kommunalt avloppsvattenreningsverk. I verket togs vattenprover från flera provpunkter som var utvalda för att täcka upp de olika stegen i reningsprocessen. MRSA i avloppsvatten identificerades dels genom odling från vattnet men också genom identificering av MRSA-DNA med hjälp av en känslig molekylderbiologisk metodik, en så kallad realtids-PCR. För att identifiera och koncentrationsbestämma *mecA* genen i avloppsvatten utvecklade vi en helt ny realtids-PCR.

Med hjälp av de utvalda metoderna kunde vi för första gången observera MRSA i avloppsvatten. Våra resultat visar att MRSA främst förekommer under ett tidigt stade i reningsprocessen och att det sker en minskning av MRSA i både antal och variationsriktedom. Det finns en antydan till att det under reningsprocessen också sker ett urval mot MRSA med

List of Papers

This doctoral thesis is based on the following papers. They are referred to in the text by their Roman numerals.


IV. Börjesson S., Mattsson A., and Lindgren PE. Genes encoding tetracycline resistance in a full-scale municipal wastewater treatment plant investigated during one year. *Manuscript*

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### Abbreviations

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<th>Description</th>
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<tbody>
<tr>
<td>BURP</td>
<td>The algorithm based upon repeat patterns</td>
</tr>
<tr>
<td>BURST</td>
<td>The algorithm based upon related sequence types</td>
</tr>
<tr>
<td>CC</td>
<td>Clonal complex</td>
</tr>
<tr>
<td>CoNS</td>
<td>Coagulase-negative staphylococci</td>
</tr>
<tr>
<td>LUX</td>
<td>Light upon extension</td>
</tr>
<tr>
<td>MRSA</td>
<td>Methicillin resistant <em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>CA-MRSA</td>
<td>Community associated MRSA</td>
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<tr>
<td>HA-MRSA</td>
<td>Hospital associated MRSA</td>
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<tr>
<td>MLST</td>
<td>Multilocus sequence typing</td>
</tr>
<tr>
<td>MSSA</td>
<td>Methicillin sensitive <em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>otr-genes</td>
<td>Oxytetracycline resistance gene</td>
</tr>
<tr>
<td>PBP</td>
<td>Penicillin-binding protein</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PFGE</td>
<td>Pulse field gel electrophoresis</td>
</tr>
<tr>
<td>PVL</td>
<td>Panton Valentine leukocidin</td>
</tr>
<tr>
<td>RPP</td>
<td>Ribosomal protection proteins</td>
</tr>
<tr>
<td>SCCmec</td>
<td>Staphylococcal cassette chromosome meC</td>
</tr>
<tr>
<td>ST</td>
<td>Sequence type</td>
</tr>
<tr>
<td>tet-genes</td>
<td>Tetracycline genes</td>
</tr>
<tr>
<td>VRE</td>
<td>Vancomycin resistant enterococci</td>
</tr>
<tr>
<td>WWTP</td>
<td>Wastewater treatment plant</td>
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</table>
1. Antibiotic resistance

Ever since the introduction of penicillin during the Second World War antibiotics have been viewed as miracle drugs. After the introduction of penicillin, isolation of new antibiotics proceeded quickly and most of the major classes were isolated during the 1940s to 1960s (169). As a result of these “miracle drugs” an accelerated decline in deaths caused by infections was seen. For example after the introduction of sulphadiazine, deaths from childbed fever caused by *Streptococcus pyogenes* decreased by 50 % in England and Wales (28). However, today the miracle may be over due to increasing antibiotic resistance in bacteria, including multi-resistant bacteria, which threatens the earlier effective treatment of bacterial infections. Antibiotic resistance has been given a lot of attention during the last two decades both within the scientific community and in public media. However, the risk of development of resistance was put forward already in the childhood of antibiotics, or to quote Sir Alexander Fleming, the discoverer of penicillin, from his Noble lecture in 1945 (6):

“It is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them, and the same thing has occasionally happened in the body.”

Just a few years later more than 50 % of the *Staphylococcus aureus* isolates were resistant to penicillin (8). The trend has not been stopped, and today more than 95 % of *S. aureus* are resistant to penicillin (119). The fact is that there are almost daily reports of bacteria that have developed resistance to common antibiotics, which they were previously susceptible to. Furthermore, to all the different antibiotic classes available, there exists at least one mechanism of resistance, and in most cases more than one (96). The biggest problem is the emergence of multi-resistant bacteria, which make treatment especially difficult, costly, and in the end maybe even impossible (170). The major reason for the increasing trend is the ongoing overuse and misuse of antibiotics worldwide. Although resistance is mainly considered to be a clinical problem the antibiotic use is not restricted to clinical settings. At least half of all antibiotics are consumed in the farming and agriculture setting (178). This use has certainly contributed to the spread and increase of antibiotic resistance.
2. Antibiotic resistance as an environmental problem

The use of antibiotics and spread of antibiotic resistance in clinical settings is a well-recognised problem, but antibiotics and antibiotic resistance as environmental problems and pollutants have largely been overlooked. This is probably due to the fact that antibiotics in non-clinical settings are generally found in concentrations well below those used therapeutically (77;81;140). However, even low levels can sustain and/or favour development and spread of antibiotic resistance in microbial communities. It is also important to realise that antibiotics and antibiotic resistance are naturally occurring and play a vital role as regulatory factors in all microbial ecosystems. In addition, several of the antibiotic resistance determinants have primary physiological roles other than giving resistance. This means that even introduction of a low concentration of antibiotics in an environment can have significant effects on the stability of the ecosystem and select for antibiotic resistance. During the last decade there has been an increasing number of reports both of antibiotic and antibiotic resistance genes in different environmental settings. It has also been described that transfer of antibiotic resistance genes can occur between bacterial strains that are unrelated evolutionary and ecologically, even in the absence of antibiotics (88). The view of the role of introduced antibiotics, antibiotic resistant bacteria and genes encoding antibiotic resistance in nature is changing. Among others, the United Nations states in their System-wide Earthwatch that research into how dispersal of antibiotics affects the natural bacterial community in non-clinical settings is essential and urgent (3). The reason is that these settings can be a potential source for spread and development of antibiotic resistance, which may find its way back into the human population.

Antibiotics are mainly introduced into the environment through two routes, human or animal treatment. However, it is important to realise that the same or similar antibiotics can be used in both human and animal treatment. Figure 1 shows anticipated routes of antibiotics and antibiotic resistance genes to the environment and potential routes back to the populations. The antibiotics are dispersed in two ways, (1) urine and faeces, or (2) direct disposal. A substantial part of all antibiotics consumed are not absorbed or metabolised by the body, but excreted in their active form in the urine and faeces. The urine and faeces are transported to wastewater treatment plants or can be used directly as manure. Direct disposal includes addition of food additives directly to the water in fish farms or treatment of crops. One large source is probably the disposal of outdated or remainders of antibiotics in household and farm
Figure 1. Potential routes for introduction of antibiotics and genes encoding antibiotics in the environment

Drains. In Germany it has been estimated that 20 - 40% of all administered antibiotics are disposed of in this way (90). In most industrialised countries a majority of households are connected to municipal sewers; in Sweden around 85% and in The United States about 75% (2;5). Wastewater treatment plants (WWTP) are therefore probably primary routes of entry for antibiotics into the environment. Several studies have also described the occurrence of different antibiotics in both untreated and treated water (81). The majority of the studies describe lower concentrations in treated water, suggesting a partial removal in WWTPs. However, it has been indicated that biodegradation does not occur for all antibiotics in the WWTPs (90). Thus, a main removal mechanism is probably through sorption to sludge (140). In most modern wastewater treatment facilities, the sludge is processed to biosolids or applied directly as fertilisers. In both treated and untreated wastewaters there are higher numbers of resistant bacteria and genes encoding antibiotic resistance compared to surface water (81). The occurrence of multiresistant bacteria, e.g. vancomycin resistant enterococci (VRE), has also been described in WWTPs (73;111). One study performed on Swedish sewage showed relatively high prevalence of VRE, which was unexpected due to the low prevalence of VRE in the human and animal population in Sweden (73). Several studies have pointed out that WWTPs can have favourable environmental conditions that increase the likelihood of
antibiotic resistance gene transfer. The potential spread of specific genes encoding antibiotic resistance in wastewater by horizontal gene transfer has also been shown (54;122;139). It is therefore essential to perform more extensive studies concerning the occurrence of antibiotic resistance and how it is affected over time and by different treatment steps in WWTPs.

3. Wastewater treatment in Sweden

Sweden was early in implementing wastewater treatment, and there was a large production boom during the 1960 - 1970s because wastewater emission into freshwaters was causing eutrophication, mainly due to the large amount of phosphorous. Today there are over 2000 publicly owned WWTPs in Sweden. During the 1990s it was realised that nitrogen in wastewater also was an environmental problem causing eutrophication in coastal areas. Therefore, during the last few years’ nitrogen removal in the Swedish WWTPs has been developed drastically. Three different treatment techniques, in different combinations, are implemented in most WWTPs today; (1) Mechanical treatment; large and heavy particles and fats are removed through coarse bar screens, sand traps and pre-sedimentation, (2) Chemical treatment; removal of phosphorus through precipitation by addition of chemical compounds, e.g. iron and aluminium, (3) Biological treatment; microorganisms, foremost bacteria, utilised for removal of the remainders of organic compounds and nitrogen. In all steps, sludge is collected and it is estimated that the WWTPs in Sweden annually produce 230,000 tons of sludge. The sludge is rich in nutrients, but may contain biological and chemical pollutants. Although faecal indicator bacteria, such as *Escherichia coli*, coliform bacteria and *Enterococci* spp., are measured for indication of bacterial content, there is no direct treatment step focusing on removal and disinfection of bacteria and possible pathogens today. (154;177)

3.1 The WWTP Ryaverket, Gothenburg, Sweden

The municipal WWTP Ryaverket in Gothenburg city is one of the largest in Northern Europe, receiving wastewater from nearly 830,000 person equivalents, with an average daily flow of 350,000 m³. It was taken into operation in 1972 and was rebuilt for biological nitrogen removal during 1995 – 1997. The wastewater is collected from five municipalities; Ale, Gothenburg, Härryda, Kungälv, Mölndal and Partille, and around 10 % of the total wastewater are from industry, health-care and public administration.
Like all other treatment plants in Sweden, Ryaverket utilises mechanical treatment, but also implements both chemical and biological treatment. In addition to removal of phosphorus, the plant is designed for biological nitrogen removal, utilising pre-denitrification in a non-nitrifying activated sludge system, and post-nitrification in a trickling filter. An overview of the complete treatment process at Ryaverket can be viewed in Figure 2. After mechanical
treatment the wastewater at Ryaverket is transferred to the activated sludge basins. In the activated sludge, bacteria is utilised for oxidation of organic particles. The first part (40 - 60 %) of the basins is anoxic which forces the bacterial community to use NO₃ for respiration resulting in NO₃ being converted to N₂, which is released directly to the atmosphere. The second part of the basins is aerobic, resulting in organic particles being oxidised through aerobic respiration. After passing the activated sludge basins, the water is pumped into sedimentation basins, where phosphorus aggregates and sludge are allowed to settle. Phosphorus is precipitated with iron sulphate, which is added prior to the activated sludge. The collected sludge is pumped back to primary settling in the mechanical treatment, while the water is separated, with about 50 % released at the Rya nabbe in the estuary of Göta River. The remaining water is mixed with ammonium rich reject water from dewatered sludge, collected mainly from primary settling, and recirculated in the system through nitrifying trickling filters (NH₄⁺ + 2 O₂ → NO₃⁻ + 2H⁺ + H₂O). The trickling filter is filled with a plastic crossflow material that provides a large surface for bacterial biofilm growth. After the trickling filters, the water is pumped back into the anoxic phase of activated sludge. In 2004, Ryaverket received on average P₉₀ of 4.4 mg l⁻¹ and N₉₀ of 27.1 mg l⁻¹ and released P₉₀ 0.4 mg l⁻¹ and N₂ 10 mg l⁻¹. The system has a hydraulic retention time of ~8 h and the activated sludge system has a solid retention time of 2-4 days. (4;138)

### 3.2 Bacterial communities and pathogens in municipal WWTPs

Bacterial communities play a vital role in wastewater treatment, since they are the ones responsible for most of the carbon and nutrient removal in WWTPs (167). In activated sludge, bacteria are used for oxidation of organic particles and the transformation of nitrate and nitrite to nitrogen (nitrification and denitrification). The bacteria in biofilms are commonly used to transfer ammonium to nitrate (nitrification).

There is no such thing as a universal bacterial flora for all WWTPs, instead studies have shown a great difference in population structure between WWTPs (20;167). The structure of the bacterial community is probably affected to a high degree by operational parameters and quality/composition of the wastewater. In Ryaverket it has been shown that the ammonium content largely affected the nature of the bacterial biofilm in the trickling filters in terms of thickness, structure, biomass content and community structure (105). However, within a WWTP the bacterial structure is often stable, at least over a 6-month period (20). Molecular typing has shown that the community has a high diversity, with high numbers of cells and...
different bacterial species (20;168). A total of 13 bacterial phyla out of the 36 described have been detected in WWTPs. The Proteobacteria is the most abundant phylum, constituting for up to 50 % of the population in WWTPs (168), and in the aeration basins in activated sludge they can account for up to 70 % (166). It is foremost $\alpha$-, $\beta$- and $\gamma$- Proteobacteria that are detected, with the $\beta$-Proteobacteria being the most frequent. Furthermore, in activated sludge, it has been shown that the $\beta$-Proteobacteria has high diversity (168). Cultivation experiments have shown that Enterobacteriaceae, *Aeromonas* spp. and *Acinetobacter* spp. can be commonly isolated (166). Besides Proteobacteria, other common phyla are Actinobacteria, Bacteroidetes, Chloroflexi and Planctomycetes.

The nitrifying and denitrifying bacteria in WWTPs have been paid a lot of attention due to their importance for reducing nitrogen levels. However, the bacteria responsible for denitrification in WWTPs are still largely unknown, but a majority probably belong to $\beta$-Proteobacteria (167;168). *Alcaligenes* spp., *Pseudomonas* spp., *Methylobacterium* spp., *Bacillus* spp., *Paracoccus* spp. and *Hypohomicrobium* spp. have all been isolated through their denitrifying ability, but it is not clear if they are representative for the *in-situ* flora. On the other hand the bacteria responsible for the nitrification process are better described. *Nitrosomonas europea* and *Nitrobacter* spp. are commonly identified in WWTPs all over the world as the main ammonium and nitrite oxidisers, but *Nitrosomonas eutropha*, *Nitrosomonas marina*, *Nitrococcus mobilis*, and phylogentic lineages of uncultured representatives are also common. In Ryaverket it was shown that the most common ammonium oxidising bacterium was *Nitrosomonas oligotropha* (104).

Besides the “normal” flora in WWTPs, a large diversity of different pathogens has been described (Table 1) (57;79;93;125;141;144;175), but the levels of pathogens are lower than those of the non-pathogenic bacteria (93). Studies showed that pathogens were detected to a higher extent in incoming water compared to outgoing, and that the treatment processes reduced their concentrations. *E. coli*, coliforms and *Enterococcus* spp. are often used as indicators for bacterial concentration and studies have shown that total coliforms was reduced by up to 6 log$_{10}$ units, *E. coli*, 1-4 log$_{10}$ units, while *Enterococcus* spp. was reduced 0.5-3 log$_{10}$ units (47;48;79). One study focusing on *Clostridium perfringens* showed a reduction by 1-2.5 log$_{10}$ units, and the authors proposed that pathogenic bacteria might survive better than the indicator bacteria (175). Studies have also shown that the treatment process reduced the
number of human viruses; astroviruses, 2-3 log_{10} units and norovirus, 0-2.5 log_{10} units (92;121). However, most of the pathogens were never completely removed (Table 1).

Table 1. Detected pathogens in wastewater

<table>
<thead>
<tr>
<th>Untreated</th>
<th>Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aeromonas hydrophilia</strong></td>
<td><strong>Aeromonas hydrophilia</strong></td>
</tr>
<tr>
<td><strong>Campylobacter coli</strong></td>
<td><strong>Campylobacter jejuni</strong></td>
</tr>
<tr>
<td><strong>Clostridium perfringens</strong></td>
<td><strong>Clostridium perfringens</strong></td>
</tr>
<tr>
<td><strong>Enterotoxigenic Escherichia coli</strong></td>
<td><strong>Enterotoxigenic Escherichia coli</strong></td>
</tr>
<tr>
<td><strong>Escherichia coli O157</strong></td>
<td><strong>Klebsiella pneumoniae</strong></td>
</tr>
<tr>
<td><strong>Listeria monocytogenes</strong></td>
<td><strong>Salmonella spp.</strong></td>
</tr>
<tr>
<td><strong>Shigella flexneri</strong></td>
<td></td>
</tr>
<tr>
<td><strong>adenoviruses</strong></td>
<td><strong>adenoviruses</strong></td>
</tr>
<tr>
<td><strong>enteroviruses</strong></td>
<td><strong>enteroviruses</strong></td>
</tr>
<tr>
<td><strong>Helminth ova</strong></td>
<td><strong>Giardia cysts</strong></td>
</tr>
<tr>
<td><strong>Cryptosporidium spp.</strong></td>
<td><strong>Giardia cysts</strong></td>
</tr>
</tbody>
</table>

*Parasitic worm, **Protozoa

4. **Staphylococcus aureus**

The genus staphylococcus was first described in 1880 (123) and is gram-positive cocci of 0.8-
1.0 μm in diameter. Under the microscope the staphylococci have a characteristic grape-like appearance, due to division in several plains (107). Today, more than 40 different staphylococcal species have been described (1), and several of them are pathogenic to humans and/or animals (71). *Staphylococcus aureus* is by far the staphylococci species that is most pathogenic to humans and is also the best studied. It can be easily separated from most other staphylococci through its ability to coagulate plasma, thanks to production of coagulase. The other staphylococci are therefore often referred to as coagulase-negative staphylococci (CoNS). The reason for the higher virulence of *S. aureus* is probably due to the wide variety
of cell-wall associated proteins and secreted toxins it possesses (22;103). This also gives *S. aureus* its capability of causing a large diversity of both benign and lethal infections in humans and animals (Table 2). (136;176) In many parts of the world *S. aureus* is the most frequent cause of hospital-acquired infections. In the USA 19 % of over 3 million bacterial isolates from inpatients were determined to be *S. aureus*, making it the most prevalent species (152). Besides being a common hospital pathogen, *S. aureus* causes infections in the community; in the USA it was the second most prevalent species from outpatient specimens. The community-acquired infections are generally minor skin infections, but there are cases of severe skin-infections and lethal haemolytic pneumonia (101). Furthermore, the total number of infections caused by *S. aureus*, both in the community and clinical settings, has increased steadily over the past decades (103).

The duality of *S. aureus* is that it is also a human commensal and has been described to be carried by ~30 % up to 70 % of the population (126). Longitudinal studies have distinguished three different carriage patterns; persistent, intermittent and non-carriage. However, some researchers make a further distinction; occasional and intermittent. Studies have shown that 12 - 30 % of the population are persistent carriers, 16 - 70 % occasional/intermittent carriers and 16 – 69 % are non-carriers. The large variation in percentages is due to differences in the studies, e.g. culture techniques, the population (carriage is connected to age, sex and ethnicity) and interpretation guidelines. The persistent carriers are colonised by a single strain, while intermittent carriers can carry different strains over time. A fit between host and the carried *S. aureus* is essential, because persistent carriers are resistant to colonisation with new strains (126;176). The genetic diversity of carriage strains of *S. aureus* is high, but no clear association of genotypes and different host attributes, such as sex, age or medical history, is

<table>
<thead>
<tr>
<th>Skin and soft tissue infections</th>
<th>Invasive infections</th>
<th>Others</th>
</tr>
</thead>
<tbody>
<tr>
<td>Botryomycosis</td>
<td>Endocarditis</td>
<td>Gastroenteritis</td>
</tr>
<tr>
<td>Carbuncles</td>
<td>Osteomyelitis</td>
<td>Toxic shock syndrome</td>
</tr>
<tr>
<td>Cellulitis/erysipelas</td>
<td>Pneumonia</td>
<td></td>
</tr>
<tr>
<td>Folliculitis</td>
<td>Sepsis</td>
<td></td>
</tr>
<tr>
<td>Fruncles</td>
<td>Septic arthritis</td>
<td></td>
</tr>
<tr>
<td>Impetigo</td>
<td>Sinusitis</td>
<td></td>
</tr>
<tr>
<td>Pyomyositis</td>
<td>Urinary tract infection</td>
<td></td>
</tr>
<tr>
<td>Scalded skin syndrome</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stye</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Diseases caused by *S. aureus*
evident (143). However, some genotypes of *S. aureus* are more prevalent among carriers. The most frequent carriage site is the anterior nose, but the exact position is still unclear. Cross-sectional surveys performed on healthy adults have reported nasal carriage rates of ~27%. Besides nasal carriage, *S. aureus* can often be isolated from the skin, perineum and pharynx. (126;176) The risk of acquiring a *S. aureus* infection increases if you are a carrier. Studies have shown that for nasal carriers the relative risk for a surgical infection increases with 1.3 - 7.0 and 80% of those with community acquired skin lesions were nasal carriers (162;176). However, among non-carriers that have acquired exogenous *S. aureus* bacteraemia the mortality is fourfold higher compared to nasal carriers (176).

### 4.1 Typing of *S. aureus*

Because of *S. aureus* status as one of the most common causes of bacterial infections, and as a commensal, it is important to understand its occurrence and distribution. It is also significant to identify if there are specific isolates or major clusters responsible for infections and colonisation (161). If there are one or a few strains, this indicates that these have genotypic and/or phenotypic factors that give a selective advantage. On the other hand, if major clusters are responsible, there should be factors that are common in the population. Accurate typing methods are therefore necessary for *S. aureus*, to identify different strains and elucidate their relationship. An optimal typing method should have high stability, reproducibility, typeability and discriminating power. Furthermore, it would be advantageous if it is user friendly, rapid and has a low cost. Several different techniques for typing, both phenotypic and genotypic, have therefore been developed for *S. aureus* over the past four decades (36). In the beginning, isolates were distinguished based on phenotypic properties. Common typing methods were antibiotic resistance typing, phage typing (resistance to a standard set of phages), serological typing (differences in antigenicity), biotyping (metabolic capabilities), bacteriocin typing and resisto-typing (resistance to various chemicals). These methods have in common that they are all limited in reproducibility, typeability and/or discriminatory power. The reason is that the expression of genes, giving rise to the resulting phenotypes, is often dependent on environmental factors, which means that unrelated strains can share phenotypic traits. The methods are also inadequate for evolutionary studies, since the genes responsible for phenotypic traits tend to evolve quickly or may be subjected to horizontal gene transfer. (36;39) All the disadvantages of phenotypic typing methods have led to the development of genotypic methods, such as plasmid analysis, southern hybridisation analysis, e.g. ribo typing (RT) and binary typing (BT); PCR-based techniques, e.g. random amplified polymorphic
DNA (RAPD), repetitive element sequence based-PCR (rep-PCR), amplified fragment length polymorphism (AFLP); pulse field gel electrophoresis (PFGE), and sequence typing, such as \( \text{spa} \) typing and multilocus sequence typing (MLST) (36). Based on the literature, the most used methods today are PFGE, MLST and \( \text{spa} \) typing (32).

4.1.1 Pulse field gel electrophoresis

PFGE is the most frequently used typing method, and many have embraced it as the golden standard for studying outbreaks and hospital transmissions. The reason is that PFGE is the most discriminative of the genotypic methods, due to the fact that it reflects genetic events that occur during a short period of time. PFGE is based on the fragmentation of the whole bacterial chromosomal DNA using restriction enzymes, often \( \text{SmaI} \). The fragmented DNA is then separated on an agarose gel using an alternating voltage field. This result in a unique band pattern, which is analysed with specially designed computer software, often using the Dice coefficient and unweighted pair grouping. However, for long-term surveillance and evolutionary studies, PFGE may be inadequate, due to the instability of the restriction sites over a prolonged period of time, and that minor genetic alterations can lead to major changes in the band pattern. The comparison of results between laboratories is also difficult or even impossible due to poorly harmonised protocols, the lack of a standardised nomenclature and the fact that the analyses of patterns are subjective. Furthermore, the PFGE is both time and cost consuming. (33;36;39)

4.1.2 Multilocus sequence typing

MLST is used for typing of almost all major human bacterial pathogens including \( S. \text{aureus} \), and more protocols are in the pipeline (159). It is based on sequencing of several housekeeping genes; for \( S. \text{aureus} \) typing sequences of approximately 500 bp are determined for seven genes; \( \text{arc, aroE, glpF, gmk, pta, tpi, and ygiL} \). The sequences are compared for each gene and different sequences are assigned distinct alleles. The alleles for all seven genes are then used to create an allelic profile and every specific profile is designated as a sequence type (ST). An \( S. \text{aureus} \) isolate assigned to ST8 has an allelic profile that looks like 3-3-1-1-4-4-3, while an isolate designated ST5 has the profile 1-4-2-4-12-1-10. Using the Based Upon Related Sequence Types (BURST) algorithm, related STs can then be joined together in clonal complexes (CC). An ST is grouped within the same CC when at least five out of seven genes have identical gene sequences/alleles. MLST has been shown to be an excellent method
with high reproducibility and is suitable for population and evolutionary studies. However, it is also expensive and laborious. (32;33)

4.1.3 spa typing

spa typing, introduced in 1996 by Frenay et al. (44) has a discriminating power that lies between MLST and PFGE, and in contrast to these methods, has been described to be suitable for studying both evolutionary events and hospital outbreaks. This fact has probably contributed to its increasing popularity over the last couple of years. Its simplicity, time efficiency and lower cost relatively to PFGE and MLST, have probably also contributed to the popularity. These advantages are due to that spa typing is a single locus sequence typing method, using only the polymorphic region X of the protein A gene, found exclusively in S. aureus. The typing is based on that the region X constitutes a number of repeats of 24 bp in length. The diversity of the region is due to the deletion and duplication of the repeats, but also in some cases point mutations. S. aureus strains are divided into spa types based on the number and arrangement of these repeats and on which repeats that are identified. As an example of spa types, one can compare t008 and t064 that have the same number of repeats, but differs in the sequence of the fourth repeat (table 3). It should be pointed out that because spa typing is more discriminating than MLST several different spa types can correspond to a single ST, but remain within an assigned CC. Furthermore, using the typing software Ridom StaphType it is possible to perform cluster analysis using the algorithm based upon repeat patterns (BURP). (32;33)

<table>
<thead>
<tr>
<th>spa type</th>
<th>Repeat succession</th>
<th>Sequence of mismatching repeats</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>t008</td>
<td>11-19-12-21-17-34-24-34-33-25</td>
<td>r21 = AAAGAAGACAAAACAAGCCCTGGC</td>
<td></td>
</tr>
<tr>
<td>t064</td>
<td>11-19-12-05-17-34-24-34-33-25</td>
<td>r05 = AAAGAAGACAAAAAGCCCTGGC</td>
<td></td>
</tr>
</tbody>
</table>

A problem with spa typing is that studies have shown that spa types on occasion violate MLST STs. This violation can occur in two ways, either that different STs, belonging to the same CC, have the same spa type, or that closely related spa types are found in distant CC (59). The reason for this problem is probably intergenomic recombination events of the spa gene. However, these events take place only rarely, and the spa gene has been shown to have both long-term in vitro and in vivo stability (86). A further disadvantage is that two parallel nomenclatures exist today, based on the works of Harmsen et al. (63), as exemplified in table
3, and Koreen et al. (86), making it difficult to compare studies. Generally, the Harmsen system is more commonly used in Europe, while Koreen is more accepted in the U.S.A. One large advantage of using spa typing is that a spa server, http://spaserver.ridom.de exists, and this is probably the world largest typing database for *S. aureus*. At the time of writing this thesis, the database lists more than 4700 spa types combined from over 270 different repeats based on over 78 000 strains from 61 countries. Furthermore, this spa server is freely accessible through the Internet and all spa types and repeat sequences can be downloaded. Submission of new repeats and spa types is possible for everyone, and the server is synchronised with the typing software Ridom StaphType (Ridom GmbH, Würzburg, Germany).

4.2 *S. aureus* in wastewater and the environment

The occurrence of *S. aureus* in the environment is scarcely studied, although failed attempts to cultivate *S. aureus* from municipal wastewater, drinking water and river surface water have been made (145;164). However, it was possible to isolate *S. aureus* from hospital wastewater (145). There have also been attempts to detect *S. aureus* using molecular methods in samples collected from municipal WWTPs, both from treated and untreated wastewater, but *S. aureus* was not detected (93;144;146). Taken together, these results indicate that *S. aureus* has low prevalence in wastewater. However, there are indications that *S. aureus* can be viable in wastewater and is more resilient than earlier believed. Cultivation experiments have shown that *S. aureus* can be sustained on culture plates supplemented with wastewater for up to 80 days, but they were not able to grow (45). Furthermore, one study made observations indicating that *S. aureus* can enter a viable but un-cultivatable state in wastewater (122). This study also described that *S. aureus* in vitro has the capability of transferring plasmids, harbouring the aminoglycoside resistance genes $\text{aac(6')-Ie+aph(2'\prime)}$ in wastewater. In addition, other groups have been able to cultivate *S. aureus* from bioaerosols in WWTPs, at least during the winter period (43). Studies have also indicated that *S. aureus* can survive the treatment process in a WWTP, because an increase in prevalence of *S. aureus* infections has been reported among residents living in proximity to areas fertilised with sewage sludge (97).
5. Methicillin resistant *S. aureus* (MRSA)

MRSA, which is resistant to all clinically used β-lactam antibiotics, is the reason for increasing numbers of *S. aureus* infections in hospitals and in the community. Besides higher infection rates MRSA is also responsible for higher cost and mortality in the society. Methicillin, a semi-synthetic penicillin, originally named celbenin, is no longer in use, but the definition MRSA remains. Methicillin was introduced in 1959 as a way to battle the increasing number of β-lactamase producing *S. aureus* strains. However, just two years after the introduction the first case of “celbenin” resistant *S. aureus* was reported (75), and in 1967 the first reports of multi-resistant MRSA came, in Australia, Denmark, England, France, India and Switzerland (51). During the 60s and 70s a swift dissemination of a specific multi-resistant clone, the phage type 83A, occurred in Europe. In Denmark 15 % and in Zürich, Switzerland, 20 % of all clones belonged to this phage type, but in the late 70s and early 80s the occurrence decreased to 2 % in Denmark and 3 % in Zürich. The reason for the decrease is unknown, but it was probably influenced by changes in antibiotic prescriptions and control policies. In the 1980s, when rapid increases in gentamycin resistant MRSA was reported in USA, United Kingdom and Ireland, the concern for MRSA was renewed (51). Today, MRSA has risen to one of the most frequent causes of hospital infections worldwide. In the USA ~34 % of all *S. aureus* were described as MRSA in 1998 – 2002; Central Europe 9 %, in 1995; Europe 26 % and Latin America 35 %, in 1997-1999; South Africa 40 %, Japan 67 % and Australia 22 % in 1998-1999. However, in Europe there are big geographical differences (Fig 3) with less than 3 % in Scandinavia, Iceland and Netherlands compared to over 30 % in

![Figure 3. The prevalence of MRSA among blood isolates of *S. aureus* in Europe 2006 (7)](image-url)
southern Europe, UK and Ireland. One study compared over 3000 MRSA isolates from Asia, Europe, Latin America and North America using different typing techniques (36). It was found that six clones of MRSA were spreading worldwide suggesting that only a few epidemic clones exist. However, there were also clones, with high diversity, dominating in a single area or hospital and from only one or a few patients. In contrast to the 70s and 80s the increasing trend is not due to a specific clone, and it appears not to secede any time soon. Furthermore, the increase in MRSA includes the low prevalence areas, Scandinavia, Iceland and Holland, e.g. in Sweden there were 1128 cases of MRSA blood isolates in 2007 compared to 327 cases in 2000 (155).

5.1 β-lactams and resistance mechanisms to β-lactams

The β-lactam antibiotics are extensively used clinically, and in Sweden they constitute ~50 % of the antibiotics administered in the community and hospital care (155). They are broad-spectrum bactericidal agents and have low toxicity to eukaryotes (49). Furthermore, it is the largest class of antibiotics and most of them are semi-synthetic compounds. The class is defined by the β-lactam ring in their chemical structure (Fig 4) and is roughly divided into four groups: penicillins (narrow to extended spectrum), cephalosporins (1st - 4th generation), carbapenems and monobactams. The exact mechanism by which the β-lactams kill the bacteria is not completely understood, but they affect the cell wall biosynthesis (62;171). The rigidity of the bacterial cell wall comes from peptidoglycan, a complex of covalently cross-linked peptide and glycan strands (Fig 5A). The cell wall synthesis includes ~30 enzymes and is performed in three stages. β-lactams inhibit the third and final stage, the completion of the cross-link, performed by transpeptidases. The inhibition is due to that β-lactams act as pseudosubstrates and acylate the active site of the transpeptidases (Fig 5B), which in the end leads to cell-lysis. The transpeptidases are therefore also termed penicillin-binding proteins (PBPs). The PBPs are found in all bacteria and in addition to the transpeptidases, there are other related PBPs.

\[
\text{Figure 4. The β-lactam ring}
\]
Figure 5. The inhibition of cell-wall synthesis by β-lactams A. Covalent Cross-linking of peptidoglycan chains B. Binding of penicillin to transpeptidases (penicillin-binding protein). Modified from Walsh (171).

Bacteria have evolved three major ways in which they avoid the bactericidal effect of β-lactam antibiotics: (1) Production of β-lactamases, which are enzymes that hydrolyse the β-lactam ring, rendering the antibiotic inactive, (2) Altered PBPs with low affinity for β-lactams, (3) Lowered or lack of expression, due to mutations, of outer membrane proteins in gram-negative bacteria, which leads to difficulty for the β-lactams to access the PBPs (12).

The first described resistance to β-lactams was the production of penicillinase by *E. coli*, and penicillinase was also the first resistance mechanism described for *S. aureus*. To date, over 530 β-lactamases have been reported and the genes encoding them (*bla* genes) are located on either the bacterial chromosome, plasmids, transposons or integrons (12;180). There are ways to overcome the β-lactamases; (1) finding inhibitors/inactivators, and (2) to develop or find new β-lactam antibiotics that are not hydrolysed or hydrolysed poorly by the β-lactamases.

The gram-positive bacteria have remained susceptible to inhibitors (16), but the introduction of semi-synthetic β-lactams, such as methicillin, cephalosporins and carbapenems, led to the evolution of PBPs with low or no affinity for β-lactams. For gram-positive bacteria, altered
PBPs are the most important resistance mechanism for β-lactams, while the gram-negative rely more on β-lactamases (12;16). The mecA gene, responsible for β-lactam antibiotic resistance in MRSA, is the most described and studied example of alternative PBP.

5.2 The mecA gene and the staphylococcal cassette chromosome mec (SCCmec)

The resistance to β-lactams in MRSA is the result of the 2.1 kb mecA gene that encodes a 78 kDA PBP dubbed PBP2a (alternatively PBP2') with low affinity for binding the β-lactams (17;32). Compared to the four native PBPs, the PBP2a appears to be a relatively poor transpeptidase, taking over only after the others have been saturated, and it also requires a native transglycosylase to function. The mecA gene is regulated by the neighbouring genes mecI and mecRI/ΔmecRI. The mecI gene codes for the repressor and mecRI/ΔmecRI codes for a trans-membrane β-lactam sensing signal transducer (32). Besides mecI and mecRI, other genes can influence the expression of mecA, for example blaRI and blalI, which are analogous to mecI and mecRI, or the factors essential for methicillin resistance (fem) genes, femA-F, femR and femX, that encode peptidoglycan modifying enzymes (17). Furthermore, genes that are part of the native genome and partake in cell-wall biosynthesis and turnover, most likely also play a role for β-lactam resistance (109).

The only vector identified for the mecA gene is the staphylococcal cassette chromosome mec (SCCmec), which has not been identified in any other bacteria except staphylococci (61). The SCCmec appears to be a well-developed vessel for genetic exchange, believed to occur through horizontal transfer, but the exact mechanism is unclear. The fact that it is transferred frequently is supported by species independent conservations (61). The distribution of SCCmec among S. aureus strains is not even, instead they are found more frequently in five out of eleven identified MLST CC (40). The reason for the uneven distribution is not known, but it may be due to that these strains are more virulent and selection pressure from antibiotic treatment may have forced them to retain SCCmec. Compared to other genes in the S. aureus genome the SCCmec appear to have been acquired relatively recent (69).

The SCCmec is introduced in the S. aureus genome at a specific site (attBSCC) at the 3’ end of an open reading frame with unidentified function (orfX) (33;34;61). The specific integration, and excision, of SCCmec into the genome is executed by the cassette chromosome recombinases (ccr), of the invertase/resolvase class, which is found in all SCCmec. In MRSA three genes have been identified that code for the ccr, ccrA and ccrB with four allotypes, and
The mecA gene itself is located on a structure termed the mec complex. Besides mecA, the genes responsible for regulation of transcription, mecl and mecRI/ΔmecRI, as well as insertions sequences, IS431 and IS1272, are found in this complex. Five different classes (A – E) of the complex have, to date, been defined based on the combination of mecl, mecRI, ΔmecRI and insertion sequences (Table 4). Of these the classes, A - C are the most common.

Table 4. The classes of the mec complex

<table>
<thead>
<tr>
<th>Class</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>mecl-mecRI-mecA-IS431</td>
</tr>
<tr>
<td>B</td>
<td>IS1272-ΔmecRI-mecA-IS431</td>
</tr>
<tr>
<td>C</td>
<td>IS431-ΔmecRI-mecA-IS431</td>
</tr>
<tr>
<td>D</td>
<td>ΔmecRI-mecA-IS431</td>
</tr>
<tr>
<td>E</td>
<td>ΔmecRI-mecA-IS431</td>
</tr>
</tbody>
</table>

In addition, to the two essential components, the ccr- genes and the mec-complex, SCCmec contain junkyard (J) regions. These J-regions are made up of non-essential components, but additional resistance genes can be found here. There are three J-regions; (1) J1, between the genome right junction and the ccr genes, (2) J2, between the ccr and the mec complex, and (3) J3, from the mec complex to the orfX. Based on the composition of the ccr genes and the mec complex, the SCCmec is divided into seven types, ranging in size from 20.9 to 66.9 kb (Fig 6) (32), and based on variations in the J-regions these types can be further divided into subtypes. The SCCmec types have been named using roman numerals, I-VII, based on the

Figure 6. Comparison of the SCCmec types I-VII described in MRSA Deurenberg and Stobberingh (32).

order in which they were described. Among MRSA isolates worldwide, the SCCmec IV dominates and is at least twice as common as any other type. The SCCmec IV also dominates
Carrying SCCmec is thought to impose an energetic cost for the staphylococci, but the type IV does not to impose a cost in terms of growth rate, cell yield or number of cells produced per mole ATP consumed (94). This can explain why type IV is more common than the other types. The type IV is also the most variable with eight subtypes, IVa-IVh, and these variations are largely believed to be a result of higher mobility of type IV compared to the other types (32). The SCCmec I, IV, V and VII do not generally carry any other resistance genes except the mecA, but the types II and III on the other hand cause multi-resistance, due to integration of plasmids. Besides carrying resistance genes in the SCCmec MRSA can harbour resistance genes in other sites of the genome and on plasmids. (32;61).

The origin of mecA and SCCmec is not known, but there are data indicating that they originated from CoNS (51;61). The leading theory is that SCCmec elements have been introduced several times in S. aureus, due to the genetic difference of MRSA strains and that the same strains harbour different SCCmec (137). The suggested number of times that SCCmec has been introduced in S. aureus is at least 20, while the same number for S. epidermidis is around 50 times (34).

### 5.3 Hospital- and community-associated MRSA

MRSA was traditionally considered just as nosocomial pathogens, but this changed when a novel MRSA was isolated in 1993 among aborigines in Western Australia, not previously exposed to western healthcare (51). After this first report came several more about community-associated MRSA (CA-MRSA), mainly occurring in groups with high physical contact. Today, CA-MRSA is described worldwide in all type of settings, and is primarily associated with skin and soft tissue infections. Furthermore, the trend is that CA-MRSA strains are replacing the more traditional hospital-associated MRSA (HA-MRSA) strains (32). In the USA, >75 % of the MRSA cases are defined to have CA background and in Scandinavia the CA-MRSA is more prevalent then the HA-MRSA (32;148). No universal definition of what a CA-MRSA really is exist, but they are generally considered to be strains isolated in outpatient settings or from a patient within 48 hours of hospital admission, with no history of previous MRSA infection/colonisation or contact with healthcare settings the previous year (32;37). In addition, the patients should have no permanent catheters or medical devices piercing the skin. CA-MRSA is more genetically diverse compared to HA-MRSA, and appear generally to carry SCCmec IV. HA-MRSA generally harbours SCCmec I, II and III and is more resistant compared to the CA-MRSA, which mainly shows β-lactam
resistance. However, the CA-MRSA is more virulent, probably due to the presence of additional virulence factors. One such factor can be the genes \textit{lukS-PV} and \textit{lukF-PV}, which encode the subunits of Panton Valentine leukocidin (PVL). PVL is a cytotoxin that causes necrosis and leukocyte destruction through pore formation (21). It is also the most consistent, of the transferable toxins, in CA-MRSA. Among CA-MRSA harbouring \textit{SCCmec IV}, PVL has been found in 40-90 \% of the isolates, while the corresponding number among \textit{SCCmec I} and II is <5 \%. However, there is evidence suggesting that PVL is not a major virulence factor (110;165).

HA-MRSA mainly appears to constitute a handful of highly epidemic clones that share a genetic background with epidemic Methicillin-sensitive \textit{Staphylococcus aureus} (MSSA) (32;34), suggesting that their successful proliferation lays in their genetics rather than that they are MRSA. Originally, it was believed that CA-MRSA was spread from the hospital to the community, but when applying PFGE and MLST it was shown that the CA-MRSA were genetically distinct from the main HA-MRSA (113;137). It was also shown that CA-MRSA had a genetically distinct background in distinct geographic areas, or that they can originate from many different backgrounds in the same area. However, CA-MRSA clones that are more frequent than others, have been identified and they are rapidly spreading worldwide (34;60). They belong mainly to the MLST ST8, ST30 and ST80, which are the same STs that are found most frequently in MSSA isolated from the community. Although the CA-MRSA are increasing they do not dominate in the community, as the HA-MRSA do in hospitals in some parts of the world, e.g. Japan, U.S.A and U.K. (34;80).

5.4 MRSA in animals
Besides being a problem in human medicine, MRSA is an increasing problem in veterinary settings (132). MRSA was first isolated in 1972 from cows with inflammation of the udder, and has since then been identified in a variety of domestic animals (95). The MRSA from household pets is generally identical to the human HA-MRSA, indicating that the animals have been colonised through human contact. However, MRSA isolated from equines and pigs have been described to be distinct from the human MRSA (32;95). In the Netherlands, it was found that 39 \% of the pigs carried MRSA, and it has been shown that persons living or working on farms (particularly pig farms) have an increased risk of being colonised with MRSA (98). Therefore, it is possible that animals and animal farms can be significant reservoirs for known and new MRSA clones.
5.5 \(\beta\)-lactams in wastewaters

\(\beta\)-lactam antibiotics, foremost penicillins and cephalosporins, have been described to occur in untreated and treated sewage from WWTPs (25;56;173;174), but concentrations were generally low, measured in ng - \(\mu\)g per litre. One study focusing on WWTPs in Sweden, showed that penicillins and cephalosporins could not be detected in a majority of samples (100). Furthermore, the \(\beta\)-lactams were reduced in the treatment process at WWTPs by up to 100 % and detected with far lower frequency in treated sewage (56;173;174). However, the removal varies and the cephalosporin Cefalexin varied in reduction between 9 and 89 % between different WWTPs (56). In addition, the cephalosporins were generally found in higher concentrations compared to the other groups, and appeared to be more resilient to the wastewater treatment, i.e. found in higher degree and concentration in outlet (56;173;174). The penicillins on the other hand were only occasionally detected, and in very low concentrations (23;56;100;173;174). In surface waters, \(\beta\)-lactams were rarely detected and when they occurred it was at concentrations well below those in wastewater (23;25;174). The reason why \(\beta\)-lactams are not persistent in aquatic environments is hydrolysis of the chemically unstable \(\beta\)-lactam ring (25).

5.6 Methicillin-resistant staphylococci and other \(\beta\)-lactam resistance in wastewater and other environments

MRSA has not been isolated from wastewater, but methicillin-resistant CoNS has been isolated from hospital wastewater (145;164). In addition, the \textit{mecA} gene has been molecularly detected in hospital wastewater, but not in municipal wastewater, surface water or agriculture soil (including soil fertilised with wastewater) (108;145;164). However, other \(\beta\)-lactam resistant bacteria and genes encoding \(\beta\)-lactam resistance occur frequently in wastewater. Bacteria resistant to penicillins, amoxicillin and/or ampicillin have been isolated from both treated and untreated wastewater in several studies (30;41;46;52;53;131;147;181). These studies generally focused on isolation of \textit{Acinetobacter} spp., Enterobacteriaceae, \textit{Enterococcus} spp. and/or \textit{Pseudomonas} spp., and described 10 % up to 100 % of the isolates as penicillin, amoxicillin and/or ampicillin resistant. In addition, many of the isolates were described to be resistant to cephalosporin and/or resistant to more than one class of antibiotic. It has also been shown that ESBL-producing \textit{E. coli} and \textit{Klebsiella} spp. frequently occur in wastewater (114;130). The question of whether WWTPs selects for a higher prevalence of antibiotic resistance or not is still unanswered. Some studies have shown more extensive and higher frequency of antibiotic resistance in treated wastewater (41;46;147), while others have
described the opposite (53;131;181). One study focusing on quantification of the β-lactamase gene \textit{bla}TEM group showed that the concentration of the gene group were higher in WWTPs compared to natural environments, but that the wastewater treatment reduced \textit{bla}TEM concentration, both related to water volume and total-DNA, by ~0.5 log\textsubscript{10} unit (91). However, if related to total number of 16S rRNA genes an increase, by ~1 log\textsubscript{10} unit, was observed in treated compared to untreated wastewater.

Besides wastewater β-lactam, including cephalosporin, resistant bacteria have been isolated from surface water, soil and wetland sediment (10;38;58;172), and in a polluted estuary, β-lactamases genes were frequently detected (66). It has also been shown that anthropogenic influence leads to an increased prevalence of β-lactam resistance (24;38;91;172).

6. Aminoglycoside antibiotics

The aminoglycosides are rapid broad-spectrum bactericidal antibiotics, which are naturally produced by \textit{Streptomyces} spp. or \textit{Micromonospora} spp. (74;87). Examples of commonly used aminoglycosides are gentamicin, tobramycin and streptomycin. They are primarily used to treat infections caused by aerobic gram-negative bacteria, but are also active against \textit{Staphylococcus} spp., \textit{Enterococcus} spp. and some \textit{Mycobacteria} spp.. However, their usefulness is limited by their serious toxicity, e.g. nephrotoxicity and ototoxicity. The class is characterised by a backbone structure consisting of an aminocyclitol ring, which in most clinically used aminoglycosides are streptamine or 2-deoxystreptamine (Fig 8). The exception is streptomycin, which has a streptidine molecule. The ring in all aminoglycosides is saturated with amine and hydroxyl substitutions, and connected to amino sugars (aminoglycosides) through glycosidic linkages. The bactericidal effect of aminoglycosides is primarily due to
binding to the 30S ribosomal subunit rendering the ribosome unavailable for translation, which results in halting protein synthesis. In addition to their direct effect on protein synthesis, they cause membrane damage through changed membrane composition and permeability, altered cellular ionic concentrations and disturbance of the DNA and RNA synthesis.

6.1 Aminoglycosides in wastewater
Occurrence of aminoglycosides in wastewater and other environments is scarcely studied, due to the fact that the antibiotics are not persistent in the environment (72). Compared to many of the other antibiotic classes, they are more readily degraded in the environment. Furthermore, aminoglycosides are generally positively charged under acidic conditions, which can facilitate adsorption to negatively charged soil and clay particles, thus further decreasing the concentration. However, one study was able to measure the occurrence of aminoglycosides in hospital wastewater, showing concentrations from 0.4 to 7.6 µg per litre (106).

6.2 Resistance to aminoglycosides
Resistance to aminoglycosides by bacteria is mediated by three mechanisms: (1) reduction of aminoglycoside concentrations via either dedicated or general efflux pumps, (2) alteration of target for the agent and (3) enzymatic inactivation of the aminoglycoside (74;87). It should be pointed out that the same bacterial strain often utilises more than one mechanism. Reduction of aminoglycoside concentration is mainly seen in non-fermenting gram-negative bacilli, such as *Pseudomonas* spp., and among others the nodulation cell division transporters superfamily play an important role in intrinsic or acquired resistance. Alteration of target sites mainly occurs through two ways; the acquisition of genes encoding rRNA methylases, which are capable of modifying 16sRNA at positions critical for binding, and secondly, point mutations of the ribosomal target. However, out of the three resistance mechanisms enzymatic inactivation is by far the most common and widespread, with >50 enzymes already described (42;74). Most of the enzymes, and the genes encoding them, are detected in gram-negative bacteria and the genes are generally located on transferable elements, i.e. plasmids, transposons and integrons. Three types of modifications have been described and the enzymes are classified based on these mechanisms: acetyltransferases (AAC); adenylyltransferases /nucleotidyltransferases (ANT); and phosphotransferases (APH) (Figure 9A), but all three mechanisms have the same end result, reduced affinity for the aminoglycosides to the ribosomal target. The enzymes have different positions on the substrate for modification,
which in the classification is denoted by a number, with or without a primer or double primer (Figure 9B). A further subclassification can be done based on which aminoglycoside that is

![diagram](image_url)

**Figure 9A.** Modification of aminoglycoside by acetyltransferases (AAC); adenyllyltransferases/nucleotidyltransferases (ANT); and phosphotransferases (APH) B. The modifying enzymes and their substrates: A amikacin, Dbk dibekacin, G gentamicin, GmB geneticin, I isepamicin, K kanamycin, N netilmicin, S Sisomicin and T tobramycin. Modified from Dessen et al (35) and Jana and Deb (74).

modified. The genes encoding the transferases follow the same nomenclature, but can be further subclassified when different genes encode transferases with the same substrate profile. A bifunctional enzyme ACC\((6')\)+APH\((2'')\) exists that can simultaneously acetylate and phosphorylate, resulting in resistance to most clinically used aminoglycosides. The gene encoding ACC\((6')\)+APH\((2'')\) is widely distributed among pathogenic bacteria and is common and highly conserved in gram-positive bacteria such as *staphylococci*, *enterococci* and *streptococci*.

### 6.3 Aminoglycoside resistance in wastewaters and other environments

The prevalence of bacteria resistant to aminoglycosides in wastewater varies greatly between location and occasion. Studies focusing on *Enterococcus* spp. have described 0 % up to 20 % of the isolates as aminoglycoside resistant (30;46;116). Aminoglycoside resistance among *Acinetobacter* spp., *E. coli* and *Pseudomonas* spp. isolates vary from generally no resistance, up to 15 % (30;46;53;131;172). However, when aminoglycoside resistant bacteria were isolated, they were often multi-resistant, and most isolates carried more than one gene encoding aminoglycoside resistance (30;163). Studies have also described that genes encoding enzymatic inactivation, from all three classes, can be detected in wastewater (67;157;163). In addition, conjugative plasmids carrying the genes have been isolated, mainly from activated sludge in WWTPs, and have been shown to be transferred to aminoglycoside-
sensitive strains *in vitro*. It has also been indicated that the number of aminoglycoside resistant isolates increases over the treatment process in WWTPs (53;42;147), but also the contrary has been described (181).

Aminoglycoside resistant bacteria and genes encoding the resistance have been detected in soil, farm-soil, coastal and estuarine water, seawater, creek/river and lake water (13;67;84;150;156;163), and conjugative plasmids have been isolated from most of these environments. It has also been described that soil harbours several not previously characterised AAC-, APH- and ANT-genes (133).

7. **Tetracycline antibiotics**

The tetracyclines are the second most clinically used antibiotic class in Sweden and are extensively used worldwide in human and animal therapy (27;155). They are also extensively used in farming, agriculture, and aquaculture. Tetracyclines exhibit activity against a wide range of gram-positive and -negative bacteria, but also against protozoan parasites and filarial nematodes. In addition, they have non-bacterial effects such as anti-inflammation, immunosuppression, inhibition of lipase and collagenase activity, enhancement of gingival fibroblast cell attachment and wound healing (134). Tetracycline is also the cheapest class of antibiotics, and has no major side effects in humans. Examples of commonly prescribed tetracyclines for human treatment are minocycline and doxycycline.

The tetracycline molecules all contain a linear fused tetracycline core (Fig 10) and an assortment of functional groups is attached to the core (27). They are only bacteriostatic and affect the bacteria through binding to the 30S subunit of the ribosome, resulting in halted
protein synthesis. However, the binding is reversible and diluting the antibiotic can reverse the effect. Besides their effect on bacteria, they also bind to 70S ribosomes in mitochondria and weakly to the 80S ribosome in eukaryotic cells.

7.1 Tetracyclines in wastewater
Tetracyclines have been detected in both treated and untreated wastewater from WWTPs, including Swedish ones, with concentrations generally in the range 10 to 1000 ng per litre (14;15;56;78;100;174). However, other studies have described no or limited occurrence in wastewater (23;70;173). Concentrations were generally reduced, by 70 to 100 %, by the wastewater treatment process at WWTPs, but studies have also shown limited, below 30 %, to no reduction (15;56;100;174). The rate of reduction is probably influenced by removal of solids in the treatment facilities, because there are indications of strong adsorption of tetracyclines to soil, sludge, clay and sediment. (15;72). However, tetracyclines appear not to be biodegraded, but photodegradation on the other hand plays an essential role in removal (72). In rivers receiving WWTP discharge, tetracycline concentrations can be as high as 400 μg per litre in the sediment (128), compared to concentrations generally below 100 ng per litre in discharge or in receiving surface water (14;100;158;174).

7.2 Resistance to tetracycline
Resistance to tetracycline exists in all bacterial genera, but the rate of resistant bacteria varies greatly between genus, species and geographical location. Most tetracycline resistant bacteria also harbour more than one resistance gene (27;134). In total, 35 tetracycline resistance genes (tet-genes) and 3 oxytetracycline resistance gene (otr-genes) have been described (135). However, no innate difference between the tet-genes and otr-genes exists, instead the nomenclature reflects the bacterium first described to carry the gene.

Of the 38 genes, 23 encode resistance through efflux pumps, most belonging to the major facilitator superfamily (MFS) (Fig 11) (134;135). The tetracycline MFS are membrane-associated proteins, commonly residing in the lipid bilayer, that export tetracycline out of the cell against the concentration gradient by exchange of one proton. This reduces tetracycline concentration and thus protects the intracellular ribosomes. Most pumps confer resistance only to tetracycline and doxycycline, but not minocycline and tigecycline. Only four genes encoding efflux pumps, tetK, tetL, tet33 and tet38, have been detected in gram-positive
bacteria. The *tetB* gene has the widest host range among gram-negatives and it confers resistance to minocycline.

Eleven of the genes encode ribosomal protection proteins (RPP), of which *tetO* and *tetM* are the best studied. However, it is generally assumed that the other nine genes have similar mechanisms (29;135). The RPPs are soluble cytoplasmic proteins conferring a wider spectrum of resistance than efflux pumps. They have high sequence similarity to the bacterial ribosomal elongation factors EF-G and EF-Tu and display, like these factors, guanosine triphosphatase (GTPase) activity. However, they cannot substitute the EF-G and EF-Tu, but may have evolved from them. Instead the RPPs confer resistance by binding to the tetracycline inhibited ribosome at the base of the h34 protein, causing an allosteric disruption and thereby releasing the tetracycline. Exactly by which mechanism the RPPs distinguish the tetracycline inhibited ribosome is not completely understood, nor whether they actively prevent the antibiotic from rebinding. RPPs have been detected in gram-positive bacteria, anaerobes and non-enteric gram-negative bacteria, and *tetM* is the most commonly detected *tet*-gene in gram-positive pathogens.

In addition to the efflux pumps and RPPs, three genes (*tetX*, *tet34* and *tet37*) have been described to confer resistance through enzymatic modification, but they are not common (135). The *tetX* has only been described in anaerobic *Bacteroides* spp. and *tet34* only in *Vibrio* spp. isolates from marine fish, while no specific bacteria have been identified carrying
the tet37. Another gene, the tetU, exists, which confers low-level resistance with unknown functions.

The tet-genes are mainly found on highly mobile elements, i.e. plasmids, transposons, conjugative transposons and integrons, and with a few exceptions pathogens acquire their resistance by acquisition of these elements. In addition, horizontal gene transfer has been described both between different species and genera (134). The gram-positive RPPs genes are generally harboured on small plasmids, while the gram-negative efflux pump genes are found on transposons or integrons inserted in a high diversity of plasmids.

7.3 Tetracycline resistance in wastewater and other environments
Tetracycline resistant bacteria are commonly isolated from a wide range of environments such as ground water, seawater, freshwater, aquaculture, soil, feedlot, and from wastewater (11;26;115;120;124). Most studies dealing with tetracycline resistance in WWTPs has focused on isolation of Coliform bacteria, Escherichia spp., Klebsiella spp., Citrobacter spp., Pseudomonas spp., Enterobacter spp., Salmonella spp., Enterococcus spp., and Acinetobacter spp. (30;41;52;131;147;183). In untreated wastewater, the rate of resistance among the isolates ranged from 6-60 % and in treated wastewaters from 16-75 %, with most of the studies describing an increase of resistance. However, there are examples describing limited or no occurrence of tetracycline resistant isolates (46). Rates of tetracycline resistant bacteria varied greatly between different types of wastewater, for example if the WWTP receives hospital or pharmaceutical wastewater, the level of resistant bacteria reported is generally higher compared to municipal wastewater (52;55;131). High rates of tetracycline resistance have also been shown in slaughterhouse WWTPs e.g. 86 % of Enterococcus spp. being tetracycline resistant (30). It has been shown that the percentage of tetracycline resistant bacteria increases with increasing influent tetracycline concentration in a WWTP (83). The biomass also influences the rate, at least in activated sludge, where higher rates of tetracycline resistant bacteria correlated with higher biomass (82). There is conflicting evidence if effluent from WWTPs increases the rate of tetracycline resistance in receiving water with studies showing an increase (52;172) and others describing no difference between WWTPs effluent, receiving water and non-receiving water (19;131).

Using a microarray targeting, 23 genes encoding tetracycline resistances, it was shown that only the genes encoding efflux pumps could be detected in farm and garden soil (124). The
A gene was detected in all soils investigated, with other common genes being tetC and tetD. In animal faeces, the RPP genes were more abundant with no single efflux gene frequently detected. In WWTPs, genes encoding efflux pumps and RPP could both be continuously detected over the treatment process, with tetA detected in all samples (11). In addition, tetA was the only gene detected in lake water. Concentration of genes tetG, encoding an efflux pump, and tetQ, encoding a RPP, were also determined during the wastewater treatment process. It was shown that tetG was reduced by 1-2 log_{10} units and tetQ by 2-3 log_{10} units, related to wastewater volume. If gene copies instead were related to total-DNA, the reduction was lower or did not occur. When tetA-E were investigated in urban and hospital wastewater tetA, but none of the other genes, was detected in all samples (55). From seawater samples collected in close proximity to outflow of WWTPs, the genes tetA, tetC, tetE, tetK and tetM could be detected (120). In sediment samples, collected from rivers receiving wastewater from WWTPs and agriculture, concentrations of tetW and tetO were generally higher compared to a river with low anthropogenic affect (128).

Tetracycline resistant bacteria and genes encoding the resistance are common in wastewater lagoons connected to animal confinement facilities and/or feedlots, with a 100% detection frequency in all studies (26;85;127;149;151). Both genes encoding RPP and efflux pumps were detected and quantified in the lagoons, but concentrations of RPPs were two to seven times higher (85;151). Genes commonly detected and/or had high abundance in the lagoons were tetM, tetA and tetB. It has been shown that the concentration of both gene types increased with higher usage of tetracycline in the feedlots (127;149), but that the RPP genes increased more drastically (127). However, only weak correlation of gene concentrations to measured tetracycline concentrations in the lagoons were detected. Tetracycline resistant bacteria and the genes encoding resistance were also frequently detected in groundwater wells and surface water in close vicinity to the animal settings (26;85;151). However, the concentration of the genes was much lower, and genes encoding efflux pumps had higher concentrations than the genes encoding RPPs (85). The rates of resistant bacteria were also lower e.g. 79 % of the Enterococci being tetracycline resistant in the lagoons as compared to 15 % in the surface waters (151).

Another environment where tetracycline resistance is commonly detected is fresh and marine aquacultures (64;115;120;139). In these studies the genes tetA and tetB were commonly
detected both in total DNA extracted and in tetracycline resistant bacterial isolates. Some of the isolates were also able to transfer tetA and tetB in vitro through plasmid conjugation.

8. Polymerase chain reaction (PCR) and Real-time PCR

The use of molecularly based methods for detection and assessment of microorganisms are an integral part in microbiology laboratories today. An abundance of different nucleic acid based systems exists for detection and assessment of antimicrobial resistance and mechanisms, extensively reviewed by Fluit et al. (42) and Sundsfjord et al. (153), with PCR, first introduced in 1988 by Mullis and co-workers (142), as the golden standard. Compared to cultivation-based methods, PCR offers a rapid, sensitive and easy way for detection of resistance genes and is especially useful for slow-growing and uncultivable microorganisms. This is important for evaluating microorganisms in non-clinical settings, such as wastewater and soil, as it has been estimated that >99% of the bacteria are uncultivable (9). However, the PCR approach contains some limitations, e.g. it is only possible to screen for what you already know, silent and pseudogenes giving false positives exists, and you cannot directly detect resistance caused by point mutations. Furthermore, PCR detects nucleic acids rather than living cells so there is a risk of “free” nucleic acids or nucleic acids from dead cells giving false-positives (179).

The basis for PCR is the detection and synthesis of a specific DNA/RNA template (129). The template is identified with two short synthetic and sequence-specific oligonucleotides, a.k.a. primers, and the primers also acts as the initiation points for the synthesis, which is carried out by polymerases. The first and most used polymerase is Taq DNA polymerase, isolated from Thermus aquaticus, but another commonly used is the high fidelity Pfu DNA polymerase, isolated from Pyrococcus furiosus. In addition, to polymerase and primers, deoxyribonucleotide triphosphates (dNTP), which are building blocks of the synthesised DNA strands, are needed. The PCR is carried out by temperature cycling (Fig 12), starting with high temperature for separation/melting of the double stranded helical DNA molecule (denaturing), after which the temperature is lowered to let primers bind (annealing) and then increased, usually to around 72 °C, to allow the polymerase to extend the primers from the 3’ end using the dNTPs (extension). This procedure is usually repeated through 25 - 45 cycles, which will allow for an exponential increase in the amount of DNA produced, with each
newly formed product acting as a template during the remaining cycles. PCR is a sensitive method where the stability and efficiency of the reaction are affected by many parameters; DNA/RNA concentration/quality, primer concentration/quality, dNTP concentration, type and concentration of polymerase, buffer type, cycling parameters, type of tubes/wells and PCR instrument. Furthermore, after completion it requires a separation of the formed PCR-product, usually by agarose gel electrophoresis with ethidium bromide staining, to confirm that the template has been amplified and that it is of expected size.

The basis for PCR has not changed significantly since its introduction over twenty years ago, but the method has been modified and improved through the years. The most significant advancement is the introduction of real-time PCR in 1993 (68) and its development into a commercially available real-time quantitative PCR in 1996 (65). The biggest problem with conventional PCR is that it is only possible to study the end product of the reaction. In theory the PCR reaction generates copies in an exponential fashion with a doubling in each cycle, but this is only true if the PCR function with 100% efficiency. During the later cycles, the PCR will not generate products in an exponential fashion. It will instead enter a plateau phase due to limitations in the polymerase, primer concentrations and dNTP concentrations. Real-time PCR circumvents this problem by using fluorescent dyes that bind to the amplified DNA in each cycle (50;89;160). The fluorescent signal is measured in each cycle and is directly proportional to the amplified DNA, making it possible to follow the PCR in “real time”. This makes it possible to study the amount of PCR product during the exponential phase and to extrapolate back to determine the original amount of template. One must be aware that during the initial cycles the signal will be weak and cannot be distinguished from the background, but as the template is amplified the signal exponentially increase (Fig 13). The time the signal
is amplified over the background depends on the initial amount of template, with higher concentrations reaching the threshold earlier (Fig 13). In order to compare different runs, the background fluorescence is subtracted from each separate run; a baseline is set (Fig 13).

Furthermore, separate runs are only comparable in the exponential phase, so a threshold must be set for the runs where all are above the baseline, in their exponential phase and parallel in growth. The number of PCR cycles that are required to reach this threshold is called the $C_t$ value. Using a serial dilution of pure DNA with known concentrations the efficiency of the PCR reaction can be estimated from a standard curve (Fig 14). The $C_t$ values of the diluted standards will be plotted against the logarithm of the standards concentration, dilution factor or number of template copies. The efficiency of the PCR corresponds to the slope of the curve, with a –3.33 slope corresponding to 100% efficiency. Using the standard curve it is also possible to determine the concentration in unknown samples, by using the determined equation of the curve and a $C_t$ value for an unknown sample. This approach is often referred to as absolute quantification or standard-curve quantification. An alternative is relative...
quantification, where, simply put, the determined Ct for the template of interest is compared to the Ct of a reference run simultaneously with the template. The reference must not vary in concentration or expression, with the efficiency of both reactions as equal as possible and preferably above 90% (50;89;160). In addition, to offer the possibility to follow the PCR in real-time and to quantify DNA/RNA, real-time PCR also eliminates the need for post-PCR processing, which reduces the time to obtain results and minimise the risks for carryover contamination.

Today a wide range of different fluorescence chemistries offered by different manufacturers exists (50;89;160). The chemistries can be broadly categorised as, (1) Non-specific DNA binding dyes/fluorophores, e.g. SYBR® green and Ethidium bromide, (2) Specific hybridisation probes, a probe is a extra oligonucleotide designed to anneal in a position “between” the primers, e.g. TaqMan™ and Molecular beacons, (3) Labelled primers, e.g. LUX™ and Amplifluor™. In some of the chemistries, the dye/fluorophore is incorporated into the final PCR product, e.g. SYBR® green or LUX™. This binding can be utilised for characterisation of the product. After the end of the PCR cycling, the temperature is increased slowly and the fluorescence continuously measured. What happens is that the PCR product starts to “melt”, resulting in the fluorescence decreasing gradually until it reaches a temperature where the double stranded DNA completely separates, resulting in an abrupt drop in fluorescence. This temperature is referred to as the melting temperature and is determined by the length and sequence of the amplified product (Fig 15). The melting temperature can be

Figure 15. Melting curve analysis. Fluorescence drops rapidly when the PCR products melt. The melting temperature is the inflection point that is easily determined as the maximum of the negative first derivate of the measured melting curve.

used to discriminate between primer-dimers, non-specific products, specific products and differences in sequences of a product (50;89;160).
In this thesis, PCR and real-time PCR are by far the most important methods. Real-time PCR is used for detection and quantification of genes encoding antibiotic resistance in wastewater environments. Several studies have earlier demonstrated real-time PCR as a fast, reliable and sensitive method for this purpose. Both PCR and real-time PCR are also applied to detect and characterise MRSA.

8.1 LUX™ real-time PCR

In this thesis we mainly worked with LUX (Light Upon eXtension) which is a relatively recent technique that only use one fluorophore that is attached to the 3’ end of either the forward or reverse primer. The labelled primer is constructed to form a hairpin-loop thus giving it fluorescence-quenching capability. The hairpin is achieved by designing a non-sequence specific tail at the 5’-end complementary to the 3’ end. To get high quenching capability the fluorophore should be incorporated within 3 nucleotides of a guanosine base. Furthermore, it is preferable to have a blunt-ended guanosine-cytosine base pairing. When the primer is incorporated into double stranded DNA the fluorophore is dequenched resulting in an increase in fluorescence (Fig 17). This technique simplifies the PCR kinetics and the 5’-tail prevents primer-dimer formation and mispriming. A further advantage is that the fluorophore is incorporated into the amplified DNA, making it possible to perform melting curve analysis. Assays are also easy to design and it is a cost-effective technique, since it does not use probes or various fluorophores. In addition, it can be used on most real-time PCR platforms.

![Figure 17. The Light Upon eXtension (LUX™) effect.](image-url)
9. Aims

The knowledge about occurrence and survival of MRSA in non-clinical environments and what roles these might play in dissemination and development are very limited. The primary aim of this thesis was to establish whether MRSA and the mecA gene occur in wastewater and how they might be affected by the wastewater treatment in a full-scale WWTP. The secondary aim was to generate knowledge about the occurrence and concentration of genes encoding aminoglycoside and tetracycline resistance in wastewater environments. It is well described that wastewater harbours aminoglycoside and tetracycline resistant bacteria and genes encoding these resistances, as described in the introduction of this thesis. However, it is scarcely studied how the gene concentrations are affected over time and by wastewater treatment. In this thesis the focus was on the genes aac(6’)-Ie+aph(2’’), mecA, tetA and tetB.

The specific aims for each individual paper were:

I. Develop quantitative real-time PCR assays, based on the LUX™ primer system, for the genes, aac(6’)-Ie+aph(2’’), mecA, tetA and tetB, to be used for analysing wastewater samples. The occurrence and concentration of these four genes were then investigated in three different types of wastewater environments.

II. Determine if the mecA gene, S. aureus and MRSA can be detected using real-time PCR in a full-scale municipal WWTP. Furthermore, to investigate the effects of the wastewater treatment process on mecA concentration and the prevalence of S. aureus and MRSA.

III. Characterise the MRSA flora in a municipal wastewater treatment plant by cultivation, how the treatment processes affects the clonal-distribution and to establish possible genetic relations to clinical isolates.

IV. Investigate how the treatment process in a full-scale municipal WWTP affects the concentrations of tetA and tetB, and determine if physiochemical parameters, bacterial and biomass load, influence the gene concentrations.
10. Sampling sites

For paper I, samples were collected from three sites representing widely different wastewater-associated environments:

(1) **Korslöt overland flow area** located in Vagnhärad in the southeast central Sweden and operated by Torsabygdens Teknik AB. It was constructed in year 2000 on a previous farmland. The area is covered with *Phalaris arundinacea* and reed *Pragmites australis* and operated all year around. From 2000 - 2003 it was flooded with wastewater from the Korslöt WWTP, and from 2003 it has been applied with landfill leachates from Korslöt recycling site. From the area, triplicate soil samples were collected at 0 – 10 cm depth, every third month from August 2004 to August 2005.

(2) **Ryaverket WWTP**, Göteborg, described in section 3.3, samples were collected from the plastic biofilm carriers in the nitrifying trickling filters. The biofilm was brushed off and suspended in 1 ml phosphate buffered saline (PBS). The samples were collected with approximately two-week intervals from December 2004 to February 2005.

(3) **County Hospital in Kalmar**, on the coast in Southeast Sweden, sludge was collected from a sewage pipeline, as grab samples, with two-week intervals from April 2003 to July 2003. The pipeline received sewage from a building containing 127 beds (72 general and urology surgery, 33 gynaecology and 22 paediatric).

**Papers II - IV** were carried out using wastewater samples collected at Ryaverket WWTP from key locations chosen to reflect the treatment processes at the plant (Fig 16). In **paper II and IV**, one 500 ml water sample was collected as a grab sample, every month from March 2006 to February 2007 at eight locations in the plant; inlet, after primary settling, activated sludge, after secondary settling, outlet, before and after trickling filter and reject water from the centrifuges. For **paper II**, two additional grab-samples were collected from Inlet in November 2007 with a two-week interval. All wastewater samples were collected in new and clean polyethylene high-density bottles. For **paper III**, water samples were collected from
four locations: inlet, activated sludge, outlet and after trickling filters (Fig 16). From each location, one 1-litre sample was collected in autoclaved Pyrex glass bottles every second week from May 5th 2008 to July 1st 2008. Inlet and Outlet samples were collected from wastewater, pooled over 24 h, while unpooled samples from activated sludge and after trickling filters were collected on site in the treatment process.

11. Design and evaluation of LUX™ real-time PCR assays for the \textit{mecA}, \textit{tetA}, \textit{tetB} and \textit{aac(6')-le+aph(2'')} genes

The main objective of paper I was to develop quantitative real-time PCR assays based on the LUX™ technique for detection and quantification of the genes \textit{mecA}, \textit{tetA}, \textit{tetB} and \textit{aac(6')-le+aph(2'')}, to be applied on samples collected from wastewater environments. At the start of this project, no well-established quantitative method was available for these genes. Quantitative methods are needed to be able to monitor prevalence of the genes in wastewater in order to study differences over time, between different environments or if concentrations are increasing due to anthropogenic effects.

Using nucleotide sequences of \textit{mecA}, \textit{tetA}, \textit{tetB} and \textit{aac(6')-le+aph(2'')} collected from GenBank (www.ncbi.nlm.nih.gov), primer-pairs suitable for LUX™ real-time PCR were designed for each gene, using the LUX™ designer (Invitrogen Corp., Paisley, UK) and the Lasergen 6.0 (DNASTAR Inc., Madison, WI, USA) (Table 5). The primer pairs were evaluated using conventional PCR on reference strains and DNA extracted from wastewater
TABLE 5. PCR primers, targeting genes coding for antibiotic resistance, designed for LUX™ real-time PCR assay. Lower-case letters at the 5’-end of primers denote the part of the primer added to give the hairpin structure. The lower-case letter t in the 3’-end of primers denotes the putative location of the dye (L in the primer name). F = forward, R = reverse (Paper I)

<table>
<thead>
<tr>
<th>Primer</th>
<th>Target gene (Amplicon size, bp)</th>
<th>Sequence (5’ - 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>aac(6´)-F1</td>
<td>aac(6´)-Ie</td>
<td>CATTACAGAGCCTTGGGAAGA</td>
</tr>
<tr>
<td>aac(6´)-R1-L</td>
<td>+aph(2´´) (364)</td>
<td>gaacatgGCCCTCGTGTAATTCATGTC</td>
</tr>
<tr>
<td>mecA-F5</td>
<td>mecA (108)</td>
<td>TGCTCAATATAAAATTTAAAACAACACTACG</td>
</tr>
<tr>
<td>mecA-R5-L</td>
<td></td>
<td>gaagtATGACGCTATGATCGATCCCAATCTAACTIC</td>
</tr>
<tr>
<td>tetA-F2-L</td>
<td>tetA (96)</td>
<td>cagccTCAATTTCTGACGGGCTG</td>
</tr>
<tr>
<td>tetA-R2</td>
<td></td>
<td>GAAGCGAGCGGGTTGAGAG</td>
</tr>
<tr>
<td>tetB-F1-L</td>
<td>tetB (101)</td>
<td>cagcaAGTGCCTTTGGATGCTG</td>
</tr>
<tr>
<td>tetB-R1</td>
<td></td>
<td>TGAGGTTGGTATCGGCAATGA</td>
</tr>
</tbody>
</table>

samples. The specificity of the primer-pairs were verified through agarose gel electrophoresis and by nucleotide sequencing. Using the designed primers, assay conditions for the real-time PCR were optimised for annealing temperature, Mg²⁺-concentration and primer concentration. The efficiencies and detection limits were then determined for each assay using serial dilutions of reference strains. Slopes (= PCR efficiencies) of the standard curves generated from the serial dilutions were as follow for each primer pair (given as mean, n ≥ 3): aac(6´)-F1+ aac(6´)-R1-L, -4.17; mecA-F5+mecA-R5-L, -3.52; tetA-F2-L+tetA-R2, -3.52 and tetB-F1-L+tetB-R1; -3.31. The detection limits were determined to aac(6´)-Ie+aph(2´´), 15 gene copies; mecA, 10 gene copies; tetA, 10 gene copies, and tetB, 5 gene copies, all per PCR reaction.

To evaluate if the developed LUX™ real-time PCR assays were suitable for quantification of mecA, tetA, tetB and aac(6´)-Ie+aph(2´´) in wastewater, they were applied on the samples collected from an overland flow area, a municipal WWTP and a hospital sewage pipeline. We were able to detect and/or quantify all genes in the three investigated environments. The results are summarised in table 6. To verify that the PCR efficiency was not compromised, due to co-extraction of inhibitory substances, serial dilutions were performed on the samples, on which the assays then were applied. It was shown that no inhibition of the PCR occurred in samples diluted more than 10⁻¹. The specificity of the assays was verified in all collected samples using melting curve analysis.
Table 6. Summary of the results of detection and quantification of the genes *mecA*, *tetA*, *tetB* and *aac*(6′)-Ie+*aph*(2′′) in three wastewater environments. **BD** = Below detection limit, **D** = Detected but not reliably quantified, **Q** = Quantified.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Overland flow area, 5 samples (genes per μg DNA)</th>
<th>Ryaverket WWTP, 5 samples (genes per μg DNA)</th>
<th>Hospital Sewage pipeline, 8 samples (genes per μg DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>aac</em>(6′)-Ie +<em>aph</em>(2′′)</td>
<td>D: 5/5</td>
<td>D: 2/5</td>
<td>Q: 8/8 (4.0x10^2 -1.4x10^5)</td>
</tr>
<tr>
<td><em>mecA</em></td>
<td>BD: 5/5</td>
<td>D: 4/5</td>
<td>BD: 7/8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Q: 1/8 (4.5 x 10^3)</td>
</tr>
<tr>
<td><em>tetA</em></td>
<td>D: 5/5</td>
<td>Q: 5/5 (1.7x10^5-8.0x10^8)</td>
<td>Q: 8/8 (2.8x10^3-6.5x10^3)</td>
</tr>
<tr>
<td><em>tetB</em></td>
<td>D: 4/5</td>
<td>BD: 5/5</td>
<td>Q: 8/8 (1.1x10^3-5.7x10^3)</td>
</tr>
<tr>
<td></td>
<td>Q: 1/5 (4.2x10^3)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The only environment where all four genes could be detected was the hospital sewage pipeline (Paper I). In addition, this was the environment with the highest concentration of all genes (Table 6). This might indicate that there is a higher selection for antibiotic resistance in this environment. The hospital pipeline probably has relatively high concentration of antibiotics compared to the other environments, which may help to sustain or select for resistance. Earlier studies have also described relatively high amounts of antibiotics in the wastewater at Kalmar County Hospital (76;99). The number of resistant bacteria that are transported to the hospital sewage pipeline is also probably higher compared to the two other environments. It was shown that the concentrations varied over time in the hospital pipeline, but no clear trend could be detected. The overland flow area is the environment with generally the lowest gene concentrations. This was expected because it treats landfill leachates and therefore probably has the lowest antibiotic pressure. Tetracycline resistance appears do be commonly detected in the environment, as described in section 8.3. So it could be expected that the genes encoding tetracycline would be detected in all environments and have the highest concentrations. The *tetA* gene had the clearest trend, lowest concentrations in the overland flow area and highest in the hospital pipeline, when comparing concentrations between the three environments. It should be pointed out that *tetA* appears to be a common gene in anthropogenic-affected environments, and compared to *tetB*, its more frequently detected (11;55;124). According to the literature *mecA* has previously only been detected in hospital wastewater. However, in this study it was shown that besides occurring in hospital wastewater *mecA* has a stable occurrence in municipal wastewater, since it was detected in
four out of five biofilm samples collected from the trickling filter in a WWTP. To our knowledge, this is also the first study to detect $aac(6')\cdot le+aph(2'')$ in municipal wastewater, but it has previously been detected in manure and soil (67).

12. Detection of the mecA gene, S. aureus and MRSA in a full-scale wastewater treatment plant

The fact that mecA was detected for the first time in municipal wastewater (paper I) raised questions whether it would be possible to detect S. aureus and MRSA in wastewater (paper II) and how the prevalence of mecA and possibly S. aureus and MRSA are affected over the treatment process and time in a full-scale WWTP. To investigate this we used the experimental set-up shown in Figure 18.

![Figure 18. Overview of the experimental procedure for molecular detection of mecA, the S. aureus specific nuc gene and MRSA.](image)

It was shown that mecA could be detected continuously over the year at all sampling sites, except in water from the rejects pumps (Table 7). S. aureus could also be detected every month in inlet, after primary settling and activated sludge. In all other sampling sites S. aureus occurred in most months, with the exception of water from reject pumps. MRSA was detected during most months in inlet, after primary settling and in activated sludge. In all other sampling sites MRSA was only occasionally detected, with the exception of after secondary settling and before trickling filter, where MRSA could not be detected.
Table 7. Molecular detection of mecA, nuc/S. aureus and MRSA, in wastewater samples collected from eight sites in a full-scale WWTP March 2006 to February 2007

<table>
<thead>
<tr>
<th>Sampling point</th>
<th>MRSA</th>
<th>S. aureus</th>
<th>+ = mecA</th>
<th>Not detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inlet</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>After primary settling</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Activated sludge</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>After secondary settling</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Outlet</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Before trickling filter</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>After trickling filter</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Reject water</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

To our knowledge this is the first report that shows occurrence of both S. aureus and MRSA in municipal wastewater. The results indicate that both S. aureus and MRSA are more resilient than previously believed and that wastewater may be a potential reservoir for MRSA. However, the treatment process appears to reduce MRSA as evidenced by that it is mainly detected in the early treatment steps. The mesophilic digestion of sludge collected from primary settling is likely to have a highly reducing effect on MRSA, as well as on S. aureus and mecA, because all three could only be occasionally detected in the water from the reject pumps. That the mecA gene is detected in all locations indicates that it mainly originates from CoNS, since CoNS often carry mecA, at least in the clinical setting (61), and since methicillin-resistant S. epidermidis have been isolated from wastewater (145). Because both mecA and S. aureus simultaneously occur in the same environment, there may be a potential for new MRSA to develop due to horizontal gene transfer. The potential for S. aureus to transfer genes encoding antibiotic resistance in wastewater has earlier been described (122).

13. Cultivation and characterisation of MRSA from municipal wastewater

In paper II it was shown that MRSA occurs in municipal wastewater and could be molecularly detected in the early treatment steps in a full-scale WWTP. However, we did not show whether MRSA is viable or how MRSA is affected over the treatment process. In addition,
the study raised some further questions: is there only one or a few strains present and are these specific for the WWTP? In order to answer these questions cultivation of MRSA was performed from four sampling sites in the WWTP (paper III), using the experimental set-up in figure 19. The clonal relationship of MRSA isolates from wastewater to the 20 most common clinical MRSA spa types in Sweden 2007 and 2008 were also determined using BURP analysis.

In total, 189 MRSA were isolated from wastewater, corresponding to 29 different spa types including six novel spa types t3950, t4013, t4100, t4102, t4203 and t4140 (Paper III). Most of the isolates, 65 %, 12 spa types, were resistant only to β-lactam antibiotics, but 21 %, 9 spa types, were defined as multi-resistant (resistant to at least two classes of antibiotics in addition to β-lactams). MRSA was isolated from inlet and activated sludge, but not from outlet or after trickling filters. Most of the isolates, 66 %, were cultivated from inlet and 19 spa types were identified in inlet compared to 15 spa types in activated sludge (Figure 20). The majority of the spa types were detected at one sampling site and one sampling occasion, but spa types t018, t127, t172 and t186 occurred in more than one sampling. In the activated sludge 40 % of
spa types were defined as multi-resistant, while in inlet only 21% were multi-resistant. PVL positive isolates, spa types t008, t019, t127 and t852, were only detected in activated sludge. The isolates were shown to harbour either SCCmec type I or IV, with 63% carrying type IV.

Based on that Sweden is a low prevalence area for MRSA and earlier studies have indicated low or no occurrence of S. aureus in wastewater, it was surprising that we were able to isolate such a great variety of spa types. However, the treatment process appears to have a negative impact on the MRSA as they can only be isolated early in the WWTP, confirming the results in paper II. Although the treatment process reduced MRSA, there are indications that it selects for more extensive antibiotic resistance and possibly more virulent strains. These indications are a higher rate of the spa types being multi-resistant in the activated sludge, and PVL positive isolates only being detected at this stage.

The results show that it is not one specific lineage of MRSA that survives in the wastewater (Paper III). Eight of the spa types, t002, t008, t015, t018, t019, t127, t186 and t790, detected in the wastewater are among the 20 most commonly reported MRSA spa types in Sweden and nine of them, t002, t008, t015, t018, t019, t067, t127, t172 and t790 are among the 30 most reported spa types in the Ridom SpaServer. The remaining spa types isolated from wastewater clustered in the same BURP-CC as the top 20 MRSA in Sweden (Fig 21). In addition, several of the wastewater isolates showed a genetic relationship, sharing spa type and SCCmec with worldwide-distributed MRSA clones (32). Together these data indicate that
the wastewater MRSA is most likely a reflection of the carriage in the community. This assumption is further supported by the fact that a large and diverse MRSA flora, changing from each sampling occasion, can be detected in the wastewater under such a short time period.

Figure 21. Dendogram based on BURP analyses of MRSA spa types isolated from municipal wastewater, and the 20 most common clinical MRSA spa types 2007 to 2008. Framed spa types = wastewater MRSA isolates (Paper III)
14. The effect of wastewater treatment on concentrations of mecA, tetA and tetB

Using the LUX™ real-time PCR assays developed in paper I, we investigated how the wastewater treatment process in a full-scale WWTP affected concentrations of mecA, tetA and tetB (paper II and IV). Furthermore, it was determined how the gene concentration correlated to faecal bacteria, e.g. E. coli and Coliform bacteria, physiochemical parameters, and biomass, using the experimental set-up in Figure 22.

It was shown that mecA, tetA and tetB could be continuously detected throughout the year at all sampling sites and that gene concentrations varied over the year (Fig 23), but no trend could be observed for the genes. When comparing the median of the gene concentrations, related to wastewater volume over the year at each sample site, it was observed that they decreased during the wastewater treatment. However, the three genes showed higher concentrations in the activated sludge compared to the other sampling sites. Higher concentration for tetA was also observed at reject water compared to at inlet. If related to biomass, i.e. total-DNA, the median over the year for mecA and tetA concentrations still decreased, but the difference between median values is smaller than those of comparison of genes and water volume. The median for mecA in inlet and after primary settling and for tetB in inlet, after primary and secondary settling and outlet were on similar levels. The median concentrations of tetA and tetB were lower in the three locations affected by biological treatment compared to the other sampled locations.
It was shown that the treatment process in a full-scale WWTP reduced concentrations of genes encoding antibiotic resistance, both related to wastewater volume and total-DNA (papers II and IV). Comparing inlet and outlet, a reduction in concentration related to volume was observed in eleven months for *tet*A and *tet*B, on average by 1.2 and 0.9 log<sub>10</sub> units, respectively, and for *mec*A in 8 months by 1.4 log<sub>10</sub> units. If gene concentrations were related to total-DNA the reductions were on average 0.3 log<sub>10</sub> units in 9 months for *tet*A, 0.2 log<sub>10</sub> units in 7 months for *tet*B and 0.5 log<sub>10</sub> units in 8 months for *mec*A. These results are supported by earlier studies showing reduction of genes encoding tetracycline resistance and genes encoding ß-lactamases in WWTPs (11;91). In the activated sludge, the gene copies related to water volume, exhibited higher concentrations compared to inlet in all months, on
average by 0.8 log\textsubscript{10} for \textit{tet}A, 0.9 log\textsubscript{10} for \textit{tet}B and 1.1 log\textsubscript{10} units for \textit{mec}A. However, when related to total-DNA the gene levels are lower in all months for \textit{tet}A and \textit{tet}B, on average by 0.7 log\textsubscript{10} units and 0.5 log\textsubscript{10} units respectively, and in 8 months, by 0.5 log\textsubscript{10} unit for \textit{mec}A, indicating that the genes have a selective disadvantage here. That gene concentrations related to volume are higher at sampling sites with a high biomass, suggests that biomass load influence the number of antibiotic resistance genes. This assumption is further supported by that \textit{tet}A concentration correlates to total-DNA concentration at most sampling sites, and that reduction of the genes when related to total-DNA is smaller compared to related to water volume. The smaller or lack of differences may also indicate that the reduction of gene copies are partly due to removal of biomass rather than direct removal of the genes. Especially for \textit{tet}A and \textit{tet}B, the reduction of biomass appears to play an essential role, since \textit{tet}A and \textit{tet}B reductions correlates to biomass reduction. However, there is still a reduction in gene concentration when comparing inlet and outlet, indicating additional mechanisms behind the reduction. Over the trickling filter, concentrations of \textit{tet}A and \textit{tet}B related to total-DNA are reduced, on average by 0.4 and 0.4 log\textsubscript{10} units (paper IV), respectively, but the \textit{mec}A concentration show an increase in 10 months by 0.4 log\textsubscript{10} units (paper II). These data suggest a selective advantage for \textit{mec}A in the trickling filter, but not for \textit{tet}A or \textit{tet}B.

Reduction of \textit{tet}A and \textit{tet}B concentrations, but not \textit{mec}A, correlates to reduction of biomass, COD and \textit{P\textsubscript{tot}} (Paper IV). This indicates that the removal of \textit{tet}A and \textit{tet}B may partly be explained by precipitation and sedimentation, as \textit{P\textsubscript{tot}} is removed by precipitation and sedimentation and COD and biomass are partly reduced by sedimentation. The notion that sedimentation is important for removal of \textit{tet}A and \textit{tet}B is further supported by that the reductions correlated to reduction of \textit{E. coli} and coliform bacteria. Sedimentation has been described to have an essential role in removal of bacteria (48;182).
15. Conclusions

- Methicillin-resistant *S. aureus* (MRSA) was for the first time shown to occur in wastewater, using both cultivation and molecular detection. The treatment processes in a full-scale municipal WWTP were also shown to reduce both number and diversity of MRSA. However, there are indications that the treatment process selects for strains with more extensive antibiotic resistance and possibly higher virulence.

- LUX™ Real-time PCR assays targeting the genes *aac*(6′)-*Ie-aph*(2′′), *mec*A, *tet*A, and *tet*B were developed and verified to be fast, sensitive and specific methods for detection and quantification of the respective resistance genes in wastewater environments. The results show that real-time PCR can be a useful tool in monitoring concentration of genes encoding antibiotic resistance, between different environments and over time, to be used for assessment of anthropogenic impact.

- By applying the developed real-time PCR assays targeting the genes *mec*A, *tet*A, and *tet*B, it was shown that the wastewater treatment in a full-scale municipal wastewater treatment plant reduced concentrations of the genes encoding antibiotic resistance. Furthermore, biomass loads appear to influence the total number of genes, and the reduction may partly be explained by removal of biomass. In addition, the reduction of *tet*A and *tet*B concentrations can partly be explained through precipitation and sedimentation in the plant.

16. Future work

In this thesis it was described that MRSA survives in municipal wastewater, and wastewater may therefore be a potential reservoir and source for MRSA. In the future it should be addressed whether new strains of MRSA can evolve in wastewater and investigated whether transfer of SCCmec can occur. However, this might be difficult to study because it has not been established what the transfer mechanisms of SCCmec are or what triggers the gene transfer. A way to get an indication whether transfer has recently occurred is to determine if
the isolated MRSA-strains can perform spontaneous excision of the SCC\textit{mec}, i.e. if the recombinases are active. The excision of SCC\textit{mec} is an essential step in a possible transfer. The SCC\textit{mec} from the isolated MRSA strains should also be further subtyped to establish if they possibly are new subtypes. It should be investigated whether MRSA has the capability to collect “new” resistance genes and virulence factors in wastewater that is not carried by the SCC\textit{mec}. Furthermore, the CoNS flora in wastewaters should be paid attention to, as well as the question if they carry SCC\textit{mec}, which SCC\textit{mec} type they harbour and whether they can transfer SCC\textit{mec}.

Although it was shown that the wastewater treatment reduced the concentration of genes encoding antibiotic resistance, the concentrations in the outlet are probably higher than in the receiving environment. It should therefore be investigated what potential impact this input has on the surrounding environments. In addition, it was shown that \textit{mecA} concentrations increased over the trickling filter in the WWTP. A more extensive study should therefore be considered for the trickling filters, for example to establish which bacteria carry \textit{mecA}, if transfer of \textit{mecA} occur and if there is an increase in bacteria carrying the \textit{mecA}. The sedimentation, precipitation and removal of biomass were shown to play an essential role in the decrease of gene concentration. It is therefore important to establish the ratio of resistant bacteria and genes encoding the resistance in the collected sludge and how concentrations are affected by the treatment of the sludge. We have also described that concentrations vary over the year in the WWTP, but since no trend could be shown it would be interesting to measure concentrations of antibiotics in the wastewater and establish if concentrations of the genes can be correlated to incoming antibiotic concentrations.
17. Acknowledgements

During the five years I’ve spent trying to be a researcher I have had the benefit of meeting a lot of interesting people, making new friends and getting help and input (sometimes much, sometimes little) from different persons. In this section I will therefore try to thank and acknowledge the people that has contributed or helped me both with the project and/or on a personal level. This will also include people I met outside the research and knew before my time as a PhD-student. If you’re not mentioned here I’m sorry, but sometimes you just forget people temporarily or completely.

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Finally remember the words of the immortal Homer Simpson:

"Phfft! Facts. You can use them to prove anything"
18. References

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