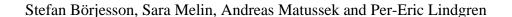
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A seasonal study of the *mecA* gene and *Staphylococcus aureus* including methicillin-resistant *S. aureus* in a municipal wastewater treatment plant

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Abstract

The spread of methicillin-resistant *Staphylococcus aureus* (MRSA), in which the *mec*A gene mediates resistance, threatens the treatment of staphylococcal diseases. The aims were to determine the effect of wastewater treatment processes on *mec*A gene concentrations, and the prevalence of *S. aureus* and MRSA over time. To achieve this a municipal wastewater treatment plant was investigated for the *mec*A gene, *S. aureus* and MRSA, using real-time PCR assays. Water samples were collected monthly for one year, at eight sites in the plant, reflecting different aspects of the treatment process. The *mec*A gene and *S. aureus* could be detected throughout the year at all sampling sites. MRSA could also be detected, but mainly in the early treatment steps. The presence of MRSA was verified through cultivation from inlet water. The concentration of the *mec*A gene varied between months and sampling sites, but no obvious seasonal variation could be determined. The wastewater treatment process reduced the *mec*A gene concentration in most months. Taken together our results show that the *mec*A gene, *S. aureus* and MRSA occur over the year at all sites investigated.

Keywords: Methicillin-resistant *Staphylococcus aureus*; *mec*A; LUXTM real-time PCR; *spa* Typing; Wastewater treatment plant; Seasonal study

1. Introduction

Antibiotic-resistant bacteria are a major threat to health care worldwide, and besides hospital and veterinary settings it has been suggested that non-clinical environments, such as wastewaters, may play a significant role in resistance development and dissemination (Martinez, 2006). Furthermore, most antibiotics are excreted from the body unchanged, and end up in the environment with continued activity (Halling-Sorensen et al., 1998). β-Lactam antibiotics have been detected in wastewater systems (Brown et al., 2006), although they are not persistent in aquatic environments due to hydrolysis of the β-lactam ring (Cha et al., 2006). Previous data have shown significantly higher numbers of antibiotic-resistant bacteria in wastewater compared to the natural environment (Kim and Aga, 2007). Little is currently known about the dynamics of antibiotic-resistant bacteria and genes encoding antibiotic resistance in wastewater treatment plants (WWTPs). It has been speculated that the wastewater treatment process may increase the proportion of resistant bacteria in outlet (Silva et al., 2006).

Staphylococcus aureus is a common pathogen in both nosocomial and community-acquired infections (Lindsay and Holden, 2004). Methicillin-resistant *S. aureus* (MRSA) is considered to be resistant to all β-lactam antibiotics, and is epidemic in hospital environments in parts of Europe (e.g. Greece, UK), Japan and the USA. In some areas, 40–60% of hospital *S. aureus* isolates are MRSA (Grundmann et al., 2006). In Sweden, less than 1% of *S. aureus* blood isolates are MRSA (SWEDRES, 2006). The gene responsible for methicillin resistance in MRSA, as well as in coagulase-negative staphylococci (CoNS), is *mec*A. The only described *mec*A vector is the staphylococcal cassette chromosome (Hanssen and Ericson-Sollid, 2006). Due to the species-independent conservation of the gene complex, it is believed that transfer of the cassette chromosome occurs frequently.

To our knowledge, MRSA has not previously been detected or isolated from wastewater systems, but methicillin-resistant CoNS have been isolated from hospital wastewaters (Schwartz et al., 2003). Previous studies have shown that *S. aureus* has a low prevalence in hospital and municipal wastewaters ([Schwartz et al., 2003] and [Shannon et al., 2007]). The *mec*A gene has been detected both in municipal and in hospital wastewaters (Börjesson et al., in press; [Schwartz et al., 2003] and [Volkmann et al., 2004]).

The aim of this study was to determine if the *mec*A gene, *S. aureus* and MRSA could be detected in a municipal WWTP, using molecular methods. Furthermore, the study investigated the effects of the wastewater treatment process on *mec*A gene concentrations and the prevalence of *S. aureus* and MRSA over time. Wastewater samples were collected monthly from the municipal WWTP, Ryaverket, Gothenburg, Sweden at eight key sites (Fig. 1) for one year.

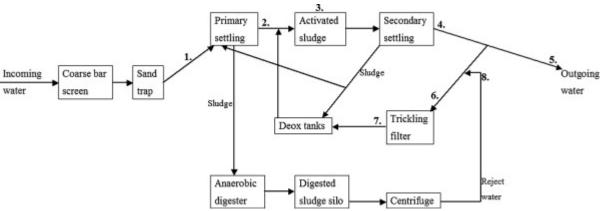


Fig. 1. Schematic drawing of the wastewater treatment process at the municipal wastewater treatment plant Ryaverket, Gothenburg, Sweden, with the main steps indicated in boxes. Sampling sites are indicated with numbers: 1. inlet, 2. after primary settling, 3. activated sludge, 4. after secondary settling, 5. outlet, 6. before trickling filter, 7. after trickling filter, and 8. water from reject pumps.

2. Materials and methods

2.1. The wastewater treatment plant and sampling

The WWTP Ryaverket, Gothenburg, Sweden, is designed for biological nitrogen removal utilizing pre-denitrification in a non-nitrifying activated sludge system and post-nitrification in a trickling filter. The plant receives wastewater from nearly 830,000 person equivalents, with an average daily flow of 350,000 m³, making it one of the largest in northern Europe. The WWTP has a hydraulic retention time of 8 h and the activated sludge system has a solid retention time of 2–4 days. In the primary settlers, heavy particles and fats are removed. The process in activated sludge system is divided in two steps, an anaerobic denitrification step and an aerobic step for decomposition of organic material. During secondary settling, sludge and phosphorus aggregates are removed. The aggregation of phosphorus is achieved by chemical precipitation with ferrous sulphate. The sludge is collected and pumped back to the primary settlers. After secondary settling half the flow is recycled to the nitrifying trickling filters. Just ahead of the trickling filters reject water from the sludge centrifuges is added in order to guarantee nitrification of the ammonium released from the sludge during sludge digestion. In the WWTP the sludge undergoes mesophilic digestion with a retention time of about 20 days. After the trickling filter, the water is mixed with return activated sludge for deoxygenation, and then led into the anaerobic step of the activated sludge. More detailed information about the treatment process at Ryaverket, and process data can be found at http://www.gryaab.se. Process data from Ryaverket during the sampling period from Mar 2006 to Feb 2007 are summarised in Table 1.

Table 1: Mean values^a \pm standard error of the process data from Mar 2006 to Feb 2007 at Ryaverket wastewater treatment plant, Gothenburg, Sweden.

	Flow (m ³ s ⁻¹)	Temperature (°C)	рН	REDOX ^b (mV)	Conductivity ^b (mS m ⁻¹)	COD (mg l ⁻¹)	Solids (mg l ⁻¹)	N _{tot} (mg I ⁻¹)	P _{tot} (mg I ⁻¹)
Inlet	4.7 ± 2.7	14 ± 3.3	7.1 ± 0.2	-232 ± 146	85 ± 25	84 ± 35	160 ± 100	27 ± 10	4.1 ± 1.7
Outlet	7.9 ± 1.2	14 ± 4.2	7.0 ± 0.2	-	-	42 ± 9.1	10 ± 5.1	9.7 ± 2.3	0.4 ± 0.1

^a Mean values of daily measurements during the sampling period.

One 500 ml water sample was collected, as grab sample, every month from Mar 2006 to Feb 2007 at eight sites in the WWTP, (1) inlet (IN), (2) after primary settling (PS), (3) activated sludge (AS), (4) after secondary settling (SS), (5) outlet (OUT), (6) before trickling filter (BTF), (7) after trickling filter (ATF), and (8) water from reject pumps (WR) (Fig. 1). Furthermore, in Nov 2007 two 500 ml water samples were collected at a two-week interval from IN, as grab samples for cultivation of MRSA. The samples were collected in new and clean 500 ml Polyethylene high-density bottles (Embalator AB, Ulricehamn, Sweden). The samples were stored at +4 °C during the sampling event in the WWTP and continuously stored at +4 °C, including the transportation time <3 h, until processing in the lab, within 24 h.

2.2. DNA extraction and quantification

The water samples, except the Nov 2007 samples, were centrifuged at 8000g for 30 min at +4 °C. The following volumes from the sampling locations were centrifuged: IN, 50 ml; PS, 100 ml; AS, 1.5 ml; SS, 150 ml; OUT, 125 ml; BTF, 125 ml; ATF, 150 ml; and WR, 8 ml (Fig. 1), the extraction volumes were determined by serial dilutions of the samples. The volume of the sample from each site was determined so that the yield of the DNA extraction should be directly proportional to the water volume of the respective environmental sample. The pellets were collected and stored at -20 °C overnight before DNA extraction. Total DNA was isolated from the pellets, using FastDNA® Spin Kit for Soil (BIO 101, Carlsbad, CA, USA), according to manufacturer protocol, and was then stored at -20 °C until molecular analyses were performed.

The reference strain *S. aureus* CCUG 35601, harbouring the *mec*A gene, was cultivated on horse blood agar plates, and for DNA extraction in LB medium, incubated overnight at 37 °C. DNA was isolated using QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA) according to manufacturer protocol.

Quantification of DNA was performed using a Pico-Green® dsDNA Quantification Kit (Invitrogen Corp., Paisley, OR, USA), essentially as described by the manufacturer.

^b Only measured in inlet.

2.3. Detection and quantification of the mecA gene using real-time PCR

For detection and quantification of the mecA gene, a real-time PCR assay based on the **LUXTM** system (Invitrogen) was used, with the primers mecA-F5: TGCTCAATATAAAATTAAAACAAACTACG-3' 5'and *mecA-*R5-L: GAAGTATGACGCTATGATCCCAATCTAACTTC-3' with an annealing temperature of 58 °C yielding a PCR product of 108 bp. (Börjesson et al., in press). The reactions were performed on an Applied Biosystems 7500 (Applied Biosystems, Warrington, UK). Reaction mixtures contained 10 µl Platinum Quantitative PCR Supermix-UDG (Invitrogen), 0.04 µl ROX Reference dye (Invitrogen), 200 nM Labelled LUXTM Primer (Invitrogen), 200 nM unlabeled primer (Invitrogen), 1 mM MgCl₂ (Invitrogen), 5–100 ng DNA, and 3.76 µl sterile distilled water to a total volume of 20 µl. The reaction conditions were: 2 min at 50 °C, 2 min at 95 °C, thereafter 40 cycles of 15 s at 95 °C, 30 s at 58 °C and 30 s at 72 °C, followed by a dissociation analysis, applying the reaction conditions: 15 s at 95 °C, 60 s at 60 °C, a 0.5 °C s⁻¹ ramping to 95 °C and 15 s at 95 °C. The real-time PCR and melting curve analyses were performed in S.D.S software version 1.3.1 (Applied Biosystems). The detection limit was determined to be 2 gene copies per PCR, based on serial dilutions of the MRSA reference strain (S. aureus CCUG 35601). In the IN sample taken on Nov 2006, quantification of the mecA gene could not be performed, probably due to enzymatic inhibitory substances in the sample.

2.4. Determination of physiochemical parameters

Physiochemical parameters were measured for IN and OUT water for all sampling months, as part of the daily routine at Gryaab Laboratory, Ryaverket, Göteborg, Sweden.

The analyses were performed according to Swedish standards (suspended solids, SS-EN 872; electrical conductivity, SS-EN 27888:1993; pH, SS 028122) and International Standards Organization (total nitrogen, ISO 11905 and 13395).

COD was measured using LANGE COD cuvette test LCK 114 (HACH LANGE LTD, Manchester, UK). REDOX and temperature were determined online using electrode measurements, REDOX; CLM60, ZK1GEL (Inventron AB, Kungsbacka, Sweden) and temperature; CLT60, Pt-100 (Endress + Hauser Conducta, Gerlingen, Germany).

Total phosphorus (P_{tot}) was determined by mixing 30 ml wastewater and 350 mg Oxisolv[®] (Merck & Co Inc., Whitehouse Station, NJ, USA), which was incubated at 200 kPa, 120 °C, 30 min and then cooled to room temperature. One PhosVerTM 3 Reagent Powder pillow (HACH LANGE LTD) was added to 25 ml of the wastewater–Oxisolv[®] solution and then incubated for 5–10 min at room temperature. The phosphorus content (P_s , mg P I⁻¹) was determined using a HACH Spectrophotometer DR/2010 (HACH LANGE LTD), program 490. To 25 ml 0.5 mg l phosphor solution (P_R) and to 25 ml distilled water (P_0) one PhosVer 3 Reagent Powder pillow was added respectively, and measured as above. P_{tot} in the sample was determined as $P_{tot} = (P_s - P_0 - P_R)$.

2.5. Detection of S. aureus and MRSA using real-time PCR

S. aureus was detected by a SybrGREEN[®] real-time PCR assay targeting the *nuc* gene (Nilsson et al., 2005) in a LightCycler instrument (Roche Diagnostics, Bromma, Sweden).

For detection of MRSA, GeneOhmTM MRSA Test (BD, GmbH, Heidelberg, Germany), designed to detect MRSA in nasal samples was applied according to manufacturer protocol. The reactions were carried out in a Smartcycler[®] (Cepheid, Sunnyvale, CA, USA).

2.6. Isolation and characterisation of MRSA strains from wastewater

150 ml of IN water was centrifuged at 8000*g* for 30 min. The pellet was resuspended in LB medium, supplemented with 3 μg ml⁻¹ cefoxitin and 8 μg ml⁻¹ aztreonam, and incubated at 35 °C for 24 h. After cultivation on MRSA selective medium (Oxoid, Cambridge, UK & Bio-Rad, Hercules, CA, USA), colonies suspected to be MRSA were subcultivated on *S. aureus* chromogenic medium (BD), Mannitol Salt Agar medium (76 mg ml⁻¹ Mannitol Salt Agar, Mast Group Ltd., Bootle, UK), and MRSA selective medium (Oxoid), and tested for DNase activity (39 mg ml⁻¹ DNase agar, Oxoid). Resistance test for cefoxitin by disc diffusion (Oxoid) on ISO-sensitest medium (31.4 mg ml⁻¹ ISO-sensitest agar, Oxoid), as well as molecular identification by *mec/nuc* PCR (Nilsson et al., 2005), was undertaken on colonies identified as *S. aureus*, and potentially MRSA. Clonal relationships were disclosed through *spa* typing (Harmsen et al., 2003).

3. Results

3.1. Detection and quantification of the mecA gene using real-time PCR

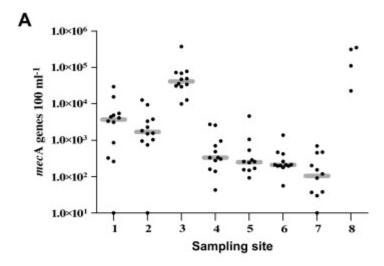
The *mec*A gene could be detected repeatedly over the year at all sampling sites, with the exception of WR (Table 2). The *mec*A gene concentrations varied over time and between the different sampling sites when related to water volume, and to biomass expressed as amount of DNA (Fig. 2). Gene concentrations were shown to decrease during the wastewater treatment process (Fig. 2A), when the median of *mec*A gene concentrations related to water volume at each sampling site over the year was compared. However, AS was noted to concentrate *mec*A, as shown from comparison to IN concentrations.

When related to biomass, *mec*A gene concentration also decreased during the treatment process (Fig. 2B), but the difference between median values is smaller than those of comparison of genes and water volume. We also observe a decrease in *mec*A gene concentration in AS compared to IN and PS, when related to biomass.

A reduction in *mec*A gene concentration ranging from 0.5 to 2.5 log₁₀ units was observed in 8 out of 12 months, when comparing IN and OUT (Fig. 3). In Aug 2006, no *mec*A could be detected in OUT. When comparing the IN and PS (data not shown), we observed a reduction in gene concentrations, 0.09–1.5 log₁₀ units in 8 out of 12 months and in Dec 2006 it could be detected, but not quantified, in PS. An increase in gene concentration was observed in the AS compared to IN for all months, ranging from 0.06 to 2.4 log₁₀ units (Fig. 3). The process in the trickling filter reduced *mec*A concentrations by 0.01–0.8 log₁₀ units, in 8 out of 12 months (Fig. 3). Furthermore, in Feb 2007, *mec*A could not be quantified, and in Aug 2006 it was below detection limit in ATF. In WR, the *mec*A gene was only occasionally detected (Fig. 2A), but gene concentration was higher than in the other sample sites on those occasions it was detected.

Table 2: Molecular detection of mecA (A), nuc/S. aureus (B) and MRSA (C). The symbol + denotes detected and – not detected. Sampling sites: (1) Inlet, (2) after primary settling, (3) activated sludge, (4) after secondary settling, (5) outlet, (6) before trickling filter, (7) after trickling filter, and (8) water from reject pumps.

Sampling site/sampli ng time	1			2		3		4			5			6			7			8				
	A	В	С	А	В	С	Α	В	С	А	В	С	А	В	С	А	В	С	А	В	С	Α	В	С
Mar 2006	+	+	+	+	+	+	+	+	_	+	+	-	+	+	_	+	+	-	+	+	+	+	+	-
Apr 2006	+	+	+	+	+	+	+	+	+	+	+	_	+	+	+	+	+	_	+	+	_	-	_	-
May 2006	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-	+	+	_	-	_	-
Jun 2006	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	-	+	+	_	-	_	-
Jul 2006	+	+	+	+	+	+	+	+	-	+	+	-	+	+	-	+	+	-	+	+	_	-	_	-
Aug 2006	+	+	_	+	+	+	+	+	+	+	_	_	_	_	-	+	_	_	_	_	_	-	_	-
Sep 2006	+	+	-	+	+	+	+	+	+	+	+	-	+	+	_	+	+	-	+	+	_	-	_	-
Oct 2006	+	+	+	+	+	+	+	+	-	+	+	-	+	+	-	+	+	-	+	+	_	-	_	-
Nov 2006	+	+	-	+	+	+	+	+	-	+	-	-	+	+	-	+	+	-	+	+	_	-	_	-
Dec 2006	+	+	+	+	+	_	+	+	_	+	+	_	+	+	_	+	+	_	+	+	_	+	+	_
Jan 2007	+	+	_	+	+	_	+	+	_	+	+	_	+	+	_	+	+	_	+	+	_	+	_	-
Feb 2007	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	-	+	+	_	+	+	+



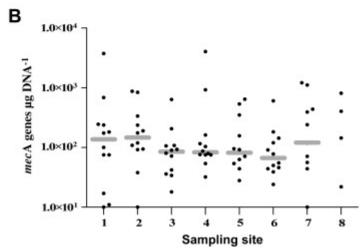


Fig. 2. Concentration of the mecA genes, determined using LUXTM real-time PCR in samples taken monthly at Ryaverket from Mar 2006 to Feb 2007. A. mecA genes 100 ml^{-1} wastewater, B. mecA genes μ g DNA⁻¹. Sampling sites: 1. inlet, 2. after primary settling, 3. activated sludge, 4. after secondary settling, 5. outlet, 6. before trickling filter, 7. after trickling filter, and 8. water from reject pumps. The symbol • indicates the concentration of mecA genes for each month. Grey dash indicates median of concentration over the year. Months when mecA was detected, but not quantified were marked as $10 \log_{10}$ genes, corresponding to the detection limit at the different sampling sites.

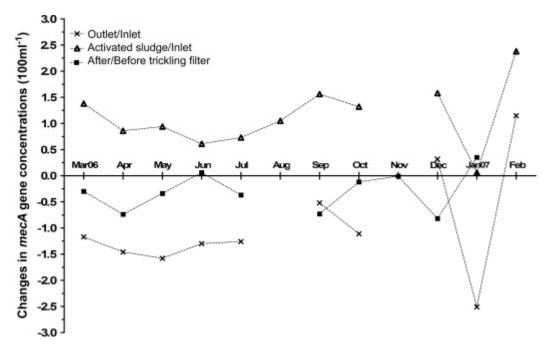


Fig. 3. The changes of the concentration of mecA gene copies 100 ml^{-1} water, determined using LUX^{TM} real-time PCR between different sampling sites. Changes are described using the \log_{10} ratios (Outlet/Inlet, Activated sludge/Inlet and After trickling filter/Before Trickling filter), positive values indicate an increase, and negative values a decrease between the sampling sites. Values for some months are not indicated in the figure due to concentrations close to, or below, the detection limit.

The *mec*A gene concentration related to biomass, expressed as amount of DNA (Figs. 2B and 4), decreased from IN and OUT in 9 out of 12 months, 0.03–2.1 log₁₀ units (Fig. 4). The gene concentration in AS decreased between 0.06 and up to 2.4 log₁₀ units, compared to IN (Fig. 4). In ATF, compared to BTF, the *mec*A gene concentration was higher in 10 out of 12 months ranging from 0.05 to 0.95 log₁₀ units (Fig. 4). Comparing IN to PS, an increase in gene concentration was detected in 9 out of 12 samples, ranging from 0.09 to 0.55 log₁₀ units (Fig. 4).

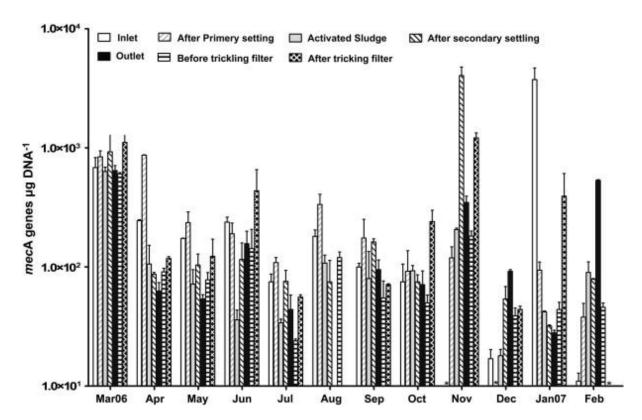


Fig. 4. The number of mecA gene copies per µg total DNA in Ryaverket, determined using LUXTM real-time PCR, in grab samples taken monthly from Mar 2006 to Feb 2007. Error bars show standard deviation for the triplicate measurements in the real-time PCR assay. The symbol (*) denotes detection under the concentration for accurate quantification.

3.2. The mecA gene concentrations related to physiochemical parameters

The *mec*A concentration in IN and OUT was related through linear regression to the following physiochemical parameters of the wastewater: temperature, pH, REDOX potential, conductivity, total chemical oxygen demand (COD), suspension, total nitrogen, and total phosphorus for each month. No correlation could be detected between the gene concentration and the physiochemical parameters.

3.3. Detection of S. aureus specific nuc gene and MRSA using real-time PCR

The *S. aureus* specific *nuc* gene was detected at all sampling sites (Table 2). In IN, PS and AS, the *nuc* gene was detected every month. In all other sampling sites, except WR, *S. aureus* could be detected in the majority of the samples.

MRSA was detected in IN, 9 months, PS, 11 months, and in AS, 6 months out of 12 months (Table 2). In SS and BTF, MRSA could not be detected. However, on two occasions MRSA could be detected in OUT, and once each in ATF and WR (Table 2). In all samples MRSA was detected, *mec*A and *S. aureus* were also detected in the same sample, there was no correlation noticed between MRSA detection and *mec*A gene concentrations.

3.4. Isolation of MRSA strains from wastewater

MRSA was cultivated on two different dates during Nov 2007 from IN. On the first occasion, *S. aureus* of *spa*-type t172 was isolated, and in the second sample, collected two weeks later, t172, t002, and t3310 were isolated.

4. Discussion

The occurrence of *mec*A, the gene responsible for methicillin resistance in staphylococci, was studied using a LUXTM real-time PCR assay, over one year at eight sampling sites (Fig. 1) in a municipal WWTP. To our knowledge this is the first seasonal study of *mec*A in a WWTP. In this study, we show that the *mec*A gene concentration varies over the year, but no seasonality (Fig. 2 and Fig. 4) or covariation with physiochemical parameters could be determined.

A reduction in gene concentration expressed per 100 ml wastewater occurred during the treatment process (Figs. 2A and 3). This is in accordance with a previous study, regarding genes encoding tetracycline resistance, which showed that the treatment process in WWTP has a reducing effect on antibiotic resistance gene concentrations (Auerbach et al., 2007). The biomass, expressed as total DNA, also decreased during the water treatment process and had the highest concentrations in the AS (data not shown). If related to biomass, a reduction of mecA concentration is still detected, but to a smaller extent (Fig. 2, Fig. 3 and Fig. 4). There was also no increase in gene concentration observed when AS to IN was compared. These results might indicate that mecA gene reduction in the WWTP, at least to some extent, is due to reduction of biomass rather than removal of the mecA gene. However, there is still a reduction in mecA gene concentration when comparing IN and OUT (Fig. 4), which might indicate a low selective advantage for carrying the mecA gene in the WWTP. Although the treatment process reduces mecA concentration, the gene is not removed in the effluent water. Other studies have shown that the mecA genes could not be detected in soil, surface water and drinking water biofilms (Börjesson et al., in press; Schwartz et al., 2003). Compared to these environments the effluent water has high concentration of mecA, indicating that WWTP may be a potential source for spread of genes encoding antibiotic resistance to other environments. However, further studies are needed to establish if or how antibiotic resistance genes can be sustained and/or transmitted within and between the indigenous bacterial flora in non-clinical environments and potential pathogens. An increase in mecA gene concentration is detected over the trickling filter (Fig. 4), which may suggest a selective advantage for carrying the mecA gene here. In the trickling filter there are high numbers of bacteria, primarily ammonia-oxidizing bacteria located in biofilms (Lydmark et al., 2007). It is known that biofilms can play an important role in dissemination, and facilitate horizontal transfer of genes encoding antibiotic resistance (Parsek and Singh, 2003). The trickling filter may therefore offer a good environment for transfer of antibiotic resistance genes.

Because both *mec*A genes and *S. aureus* simultaneously occur in the same environment (Table 2) there is potential for horizontal gene transfer giving rise to MRSA. The potential of *S. aureus* to transfer genes encoding antibiotic resistance in wastewater has been shown (Ohlsen et al., 2003). The occurrence and survival of *S. aureus* in wastewater environments have not been well studied, but the indication from both cultivation and PCR is that *S. aureus* occurs at low levels in municipal wastewaters and surface waters ([Schwartz et al., 2003], [Savichtcheva et al., 2007] and [Shannon et al., 2007]). In the present study *S. aureus* was detected to almost the same extent in OUT as well as IN (Table 2), and other groups have

been able to isolate *S. aureus* from microbial aerosols in WWTPs (Fracchia et al., 2006). Furthermore, among residents living in proximity to areas fertilised with treated wastewater,

the prevalence of *S. aureus* infections was 25 times higher than infections among hospitalised patients (Lewis et al., 2002). This indicates that *S. aureus* is more resistant in WWTP and effluent water than earlier believed.

The treatment process may reduce MRSA, as evidenced by its detection mainly in early steps in the treatment process (Table 2), e.g. in IN, PS and AS (Fig. 1). However, we cannot exclude the possibility that MRSA is present in concentrations below the detection limit of 15 genomes reaction⁻¹ (BD) at the other sampling sites, corresponding to 3.3×10^2 - 3.3×10^4 genomes 100 ml⁻¹ wastewater. That mecA is detected in all locations can indicate that the genes originate mainly from CoNS, which often carry mecA genes at least in clinical environments (Hanssen and Ericson-Sollid, 2006). In fact, methicillin-resistant CoNS have previously been cultivated from wastewater environments (Schwartz et al., 2003). The occurrence of MRSA as detected by PCR in the WWTP was verified through cultivation from IN water. Moreover, Sweden has a low MRSA prevalence in medical settings (SWEDRES, 2006). Taken together these results can indicate that sewage systems may be a potential reservoir for MRSA strains and/or healthy carriers in the community may harbour MRSA to a greater extent than earlier believed. It is important to further investigate the MRSA community in the WWTP, and to study how it is affected by the treatment process, e.g. if the process is selective for specific strains. Furthermore, it is crucial to investigate if mecA has the potential to horizontal transfer between different staphylococcal species and different S. aureus strains, thus giving rise to new MRSA strains. New techniques should be considered for reducing the levels of antibiotic-resistant bacteria in the wastewater treatment process, and the current study offers the potential to monitor the effect of these process developments.

5. Conclusions

- Using molecular methods and cultivation, MRSA was for the first time detected in a municipal activated sludge and trickling filter WWTP, but mainly in the early treatment steps, IN, PS and AS.
- The mecA gene and S. aureus could be detected throughout the year at all sampling sites.
- The wastewater treatment process reduces *mec*A gene concentrations, which can partly be explained by removal of biomass.

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