Genes encoding tetracycline resistance in a full-scale municipal wastewater treatment plant investigated during one year

Stefan Börjesson, Ann Mattsson and Per-Eric Lindgren

ABSTRACT

Tetracycline-resistant bacteria and genes encoding tetracycline resistance are common in anthropogenic environments. We studied how wastewater treatment affects the prevalence and concentration of two genes, *tet*A and *tet* B, that encode resistance to tetracycline. Using real-time polymerase chain reaction (PCR) we analysed wastewater samples collected monthly for one year at eight key-sites in a full-scale municipal wastewater treatment plant (WWTP). We detected *tet*A and *tet* B at each sampling site and the concentration of both genes, expressed per wastewater volume or per total-DNA, decreased over the treatment process. The reduction of *tet*A and *tet* B was partly the result of the sedimentation process. The ratio of *tet*A and *tet* B, respectively, to total DNA was lower in or after the biological processes. Taken together our data show that tetracycline resistance genes occur throughout the WWTP, and that the concentrations are reduced under conventional operational strategies.

Key words | LUX™ real-time polymerase chain reaction (PCR), *tet*A, *tet* B, tetracycline, wastewater treatment plant

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INTRODUCTION

Tetracyclines are cheap, broad-spectrum antibiotics, used extensively in human and veterinary medicine, as well for general antimicrobial use and growth promotion in farming and agriculture (Roberts 2003). In wastewater treatment plants (WWTPs) tetracyclines have been detected both in the influent and effluent (Karthikeyan & Meyer 2006). Bacteria resistant to tetracycline and genes encoding tetracycline resistance (*tet*-genes) are also commonly described in WWTPs (Guillaume *et al.* 2000; Auerbach *et al.* 2007; Zwenger & Gillock 2009). One study has shown that the fraction of tetracycline-resistant bacteria increased with increasing concentrations of influent tetracycline (Kim *et al.* 2007*a*,*b*). In addition, it has been suggested that *tet*-genes can be transferred between different bacterial species in wastewater environments (Rhodes *et al.* 2000). However, previous studies of *tet*-genes and tetracycline-resistant bacteria in WWTPs have mainly described their occurrence and/or concentration, but their relation to physiochemical parameters, bacterial and biomass load have not been considered in the investigations. In addition, it is poorly studied how concentrations of *tet*-genes are affected over time and by the treatment processes in WWTPs.

In this study we focus on the *tet*A and *tet* B genes, which are common in soil and water environments (Auerbach *et al.* 2007; Patterson *et al.* 2007; Börjesson *et al.* 2009*a*). It has been shown that the concentration of both genes varies between different types of wastewater environments and over time (Börjesson *et al.* 2009*a*), and one study showed that the *tet* B concentration in wastewater is connected to the use of tetracycline (Peak *et al.* 2007). Furthermore, environmental bacterial isolates have been described as transferring plasmids, harbouring *tet*A and *tet* B, *in vitro* at temperatures similar to WWTPs, ~ 15°C (Casas *et al.* 2004).

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The *tet*A and *tet* B encode resistance through efflux pumps, and genes encoding tetracycline efflux pumps are more common in the environment compared to those encoding ribosomal protection proteins, the other main tetracycline resistance mechanism (Roberts 2005; Patterson *et al.* 2007). Furthermore, *tet* B has the widest host range of the genes encoding efflux pumps among gram-negative bacteria (Roberts 2005).

The aim of the present study was to investigate how the treatment process in a full-scale municipal WWTP affects *tet*A and *tet* B concentrations during all seasonal changes over one year, using our recently developed LUX™ real-time PCR assays, previously shown to exhibit high sensitivity and specificity for determination of gene concentration in wastewater (Börjesson *et al.* 2009*a*). Furthermore, we wanted to determine in what way the concentrations of *tet*A and *tet* B are related to the concentrations of faecal bacteria and physiochemical parameters (e.g. total-phosphorus, chemical oxygen demand (COD) and biomass) and to describe and explain the eventual correlation between them. To our knowledge, this is the first long-term study to describe how the treatment process in a WWTP affects *tet*A and *tet* B concentrations during the seasonal changes over one year, and how the concentrations are related to the physiochemical parameters, bacterial and biomass load.

MATERIALS AND METHODS

The wastewater treatment plant and sampling

The Rya WWTP, Gothenburg, Sweden, receives wastewater from ~ 830,000 person equivalents, with a daily average flow of 350,000 m^3. It is designed for biological nitrogen removal, utilising pre-denitrification in a non-nitrifying activated sludge system, and post-nitrification in a trickling filter. The system has a solids retention time of 2–4 days in the activated sludge system and a total hydraulic retention time of 8–10 h. Detailed information about the treatment process at Rya WWTP, and process data can be found at http://www.gryaab.se.

One 500 ml water sample was collected as a grab sample monthly from March 2006 to February 2007 at each of the following 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. *reject water from the centrifuges* (WR) (Figure 1). The samples were collected in new and clean 500 ml polyethylene high-density bottles (Embalator AB, Ulricehamn, Sweden). During the sampling event, transportation from the WWTP to the lab (transportation time ~ 3 h) and until DNA-extraction, the samples were stored continuously at + 4°C.

DNA extraction and quantification

Wastewater samples of the following volumes were centrifuged at 8,000 *g* for 30 minutes at + 4°C: 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. The volume of water centrifuged from each sampling site was determined so as not to overload the spin-columns used in the extraction kit, and designed so that the yield of DNA extraction should be proportional to the volume of the wastewater sample. DNA was extracted using the FastDNA® Spin Kit for Soil (BIO 101, Carlsbad, CA, USA), and was stored at − 20°C until used.

Escherichia coli LH1035 A.LU1 and *E. coli* D20-16, harbouring the *tet*A and *tet* B respectively, were used as reference strains (Börjesson *et al.* 2009*a*). The *tet*A (*E. coli* LH1035 A.LU1) and *tet* B (*E. coli* D20-16) are encoded by low copy number plasmids with 1–2 plasmids per cell. The *E. coli* LH1035 has a conjugative plasmid, transferred from a bacterial strain isolated from duck faeces, which has been verified to contain the *tet*A gene (L.-O. Hedén, Lund University, personal communication). The *E. coli* LH1035 A.LU1 and *E. coli* D20-16 were cultivated on horse blood agar plates at 37°C, and one bacterial colony was transferred to LB-medium. After overnight incubation at 37°C, bacterial DNA was isolated using QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA) according to manufacturer protocol.

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

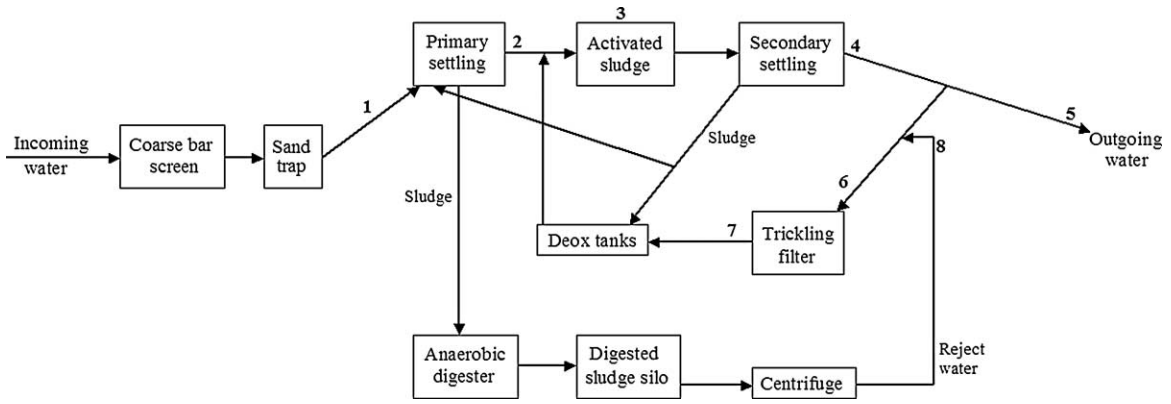

Figure 1 | Schematic drawing of the wastewater treatment process of the municipal wastewater treatment plant Ryaverket, Gothenburg, Sweden, with the main steps indicated in boxes. Sampling sites are indicated with numbers; 1. inlet, 2. after primary settling, 3. activated sludge, 4. after secondary settling, 5. outlet, 6. before trickling filter, 7. after trickling filter, and 8. water from reject pumps. Previously presented in Börjesson *et al.* (2009*b*).

Detection and quantification of the *tet*A and *tet* B genes

For detection and quantification of the *tet*A and *tet* B genes, two recently developed LUX™ real-time PCR assays, designed for quantification with high sensitivity were applied, one for each gene (Börjesson *et al.* 2009*a*). The reactions were performed in an Applied Biosystems 7500 Real-time PCR (Applied Biosystems, Warrington, UK). The reaction mixture contained 10 μl Platinum Quantitative PCR Supermix-UDG (Invitrogen Corp.), 0.04 μl ROX Reference dye (Invitrogen Corp.), 200 mM labelled LUX™ Primer (Invitrogen Corp.), 200 mM unlabeled primer (Invitrogen Corp.), 4.16 μl sterile distilled water and template DNA to a total volume of 20 μl. The following reaction conditions were applied: 2 minutes at 50°C, 2 minutes at 95°C, thereafter 40 cycles of 15 s at 95°C, 30 s at 60°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 with S.D.S software v1.3.1 (Applied Biosystems). For the IN sample of November 2006, the gene concentration could not be determined due to inhibition of the PCR-reaction. The detection limits of the real-time PCR assays were determined to eight gene copies per PCR-reaction for *tet*A, and four gene copies per PCR-reaction for *tet* B, based on serial dilution of DNA from the respective reference strain.

Quantification of total coliform bacteria and *Escherichia coli*

Detection and quantification of total coliform bacteria and *E. coli* were performed at the Laboratory of Göteborg Vatten (Gothenburg, Sweden), using the most probable number (MPN) method; colilert®-18 (IDEXX Laboratories Inc., Westbrook, ME, USA) on samples collected from April 2006 to February 2007.

Physiochemical parameters

Physiochemical parameters were measured for IN and OUT water during all sampling months, as part of the daily routine at the laboratory of Rya WWTP. 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 Organisation (total Nitrogen (N_{tot}), ISO 11905 and 13395).

COD was measured using the 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 were then incubated at 200 kPa, 120°C, 30 minutes, and cooled to room temperature. To 25 ml of the wastewater-Oxisolv® solution,

one PhosVer™ 3 Reagent Powder pillow (HACH LANGE LTD) was added and incubated 5–10 minutes at RT. The phosphorus content (P_s, mg $P\,l^{-1}$) was determined using a HACH Spectrophotometer DR/2010 (HACH LANGE LTD), program 490. To 25 ml distilled water (P_0) and 25 ml 0.5 mg l^{-1} phosphorus-solution (P_R), one PhosVer™ 3 Reagent Powder pillow was added, and measured as above. P_{tot} in the sample was calculated using $P_{tot} = (P_s - P_0 - P_R)$.

Statistical methods

The *tet*A and *tet*B gene concentrations, total-DNA, coliform bacteria, *E. coli* and physiochemical parameters were correlated with each other using Spearmans correlation coefficient, two-tailed, p-value < 0.05, in SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA).

RESULTS

Detection and quantification of *tet*A and *tet*B in wastewater

The *tet*A and *tet*B genes were detected continuously over the year at all sampling sites (Figure 2). The concentration of both genes varied over time and between the different sampling sites. No trend over the year could be observed for the genes, but the concentration of *tet*B was several fold

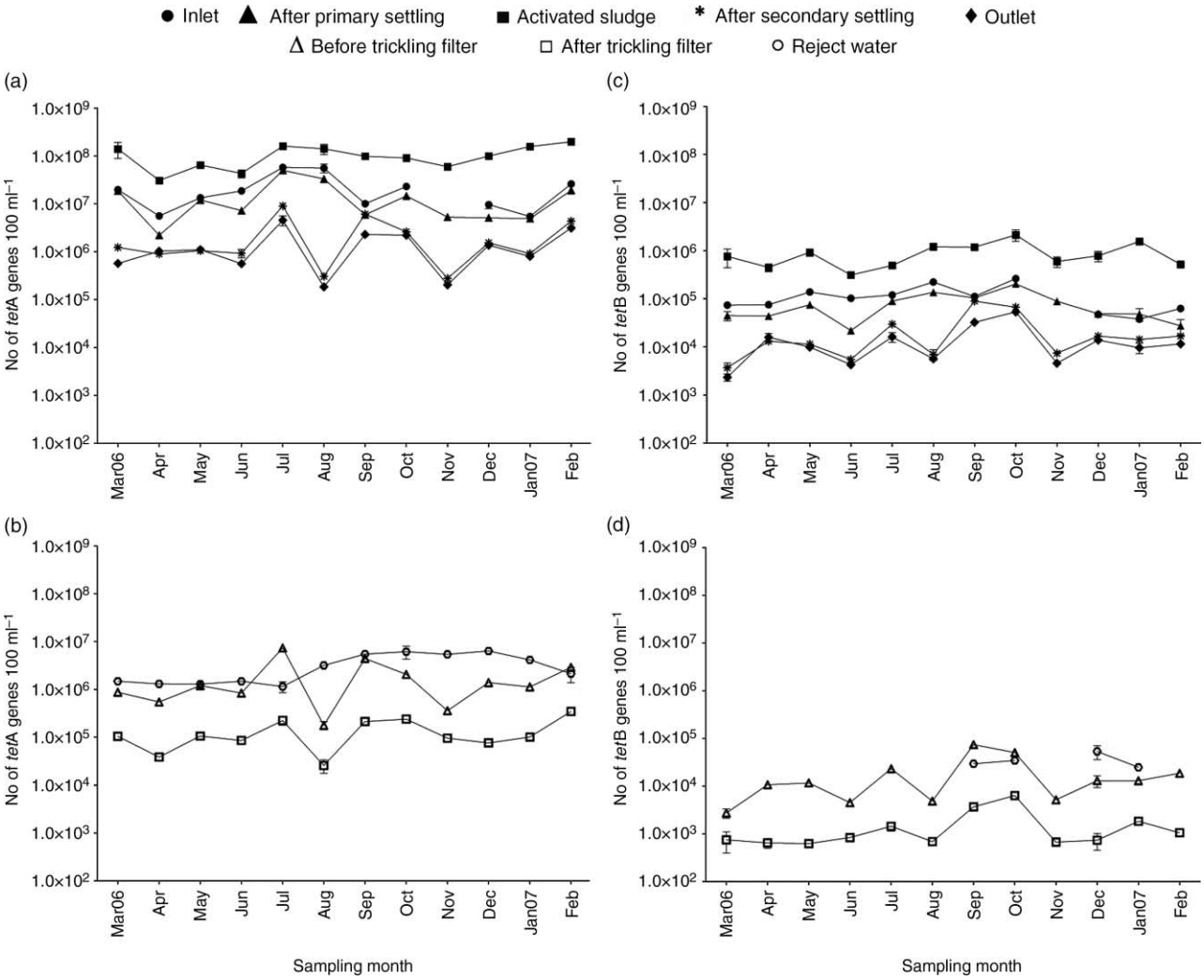

Figure 2 | Concentration of the *tet*-genes per 100 ml wastewater, at Ryaverket, determined using LUX™ real-time PCR, in grab-samples taken monthly from March 2006 to February 2007. (a-b) *tet*A; (c-d) *tet*B. Error bars show standard deviation for the triplicate measurements in the real-time PCR assay.

Table 1 | Median of *tet*A and *tet* B concentrations, determined using LUX™ real-time PCR in grab-samples taken monthly from March 2006 to February 2007 at Ryaverket

Sampling site	*tet*A genes $100\,ml^{-1} \times 10^{-2}$	*tet*A genes μg $DNA^{-1} \times 10^{-2}$	*tet* B genes $100\,ml^{-1} \times 10^{-2}$	*tet* B genes μg $DNA^{-1} \times 10^{-2}$
Inlet	160,000	7,700	890	460
After primary settling	95,000	8,100	620	470
Activated sludge	980,000	1,800	7,700	140
After secondary settling	11,000	4,300	140	380
Outlet	11,000	3,900	110	400
Before trickling filter	12,000	3,700	120	310
After trickling filter	1,000	1,500	79	170
Water from reject pumps	27,000	200	–	–

lower compared to *tet*A. When comparing the median of the *tet*A and *tet* B gene concentrations over the year at each sampling site, we observed that the concentrations decreased during the wastewater treatment process (Table 1). However, for both genes, higher medians were observed in AS than in IN. Furthermore, for *tet*A the median was higher in WR compared to BTF, ATF and OUT. Higher concentrations of both genes were observed at sites with higher amount of biomass, i.e. AS and WR.

Comparing IN and OUT, a reduction was observed in *tet*A and *tet* B gene concentrations: 0.6–2.5 and 0.5–1.6 $\log_{10}$ units, respectively (Figure 2a and c). Comparing IN and PS, a reduction was observed in 11 months, from 0.03 to 0.4 $\log_{10}$ units, for *tet*A concentration (Figure 2a), and in 9 months from 0.03 to 0.7 $\log_{10}$ units for *tet* B concentration (Figure 2c). Accumulations of *tet*A, 0.4–1.5 $\log_{10}$ units, and *tet* B, 0.5–1.6 $\log_{10}$ units were observed in AS compared to IN (Figure 2a and c)). The process in the trickling filter reduced the *tet*A and *tet* B concentrations by 0.6–1.5 and 0.6–1.3 $\log_{10}$ units, in all months (Figure 2b and d).

If related to biomass, the *tet*A and *tet* B gene concentrations varied over time and between the sampling sites (Figure 3). When comparing the median of *tet*A concentration over the year at each sampling site, we observed that the concentration decreased during the wastewater treatment process, but the decrease was smaller compared to when the *tet*A gene concentration was related to water volume (Table 1). The median for *tet* B concentrations was on similar levels in IN, PS, SS and OUT. The smaller difference or lack of difference, between median of gene concentrations was very likely a consequence of the biomass reduction from the wastewater treatment process. The median concentrations of *tet*A and *tet* B are lower in the three locations affected by biological treatment (AS, ATF and WR) compared to the remaining locations.

Reduction in gene concentration was observed over the treatment process during most months (Figure 3). In IN compared to OUT, a reduction was observed in 9 months, 0.1–0.7 $\log_{10}$ units, for concentrations of *tet*A and in 7 months, 0.1–0.4 $\log_{10}$ units, for *tet* B. When AS is compared to IN, reduction in *tet*A, 0.5–1.1 $\log_{10}$ units, and *tet* B, 0.3–0.9 $\log_{10}$ units, was observed in all months (Figure 3).

Correlation of *tet*A and *tet* B gene concentrations to biomass, faecal bacteria and physiochemical parameters

In most samples, the concentration of *tet*A, expressed per water volume, exhibited a significant correlation to *tet* B concentration (expressed per water volume), biomass (expressed as total-DNA concentration) and coliform bacteria concentration (Table 2). The *tet* B concentration correlated only occasionally to biomass, coliform concentration and *E. coli* concentration. The MPN-values of coliform bacteria and *E. coli* exhibited a significant correlation to each other in all sampling sites except in ATF.

The reduction in $\log_{10}$ units, of *tet*A and *tet* B in IN compared to OUT correlated significantly to each other (Table 3). The *tet*A and *tet* B reductions, in IN compared to OUT, significantly correlated to the reduction of total

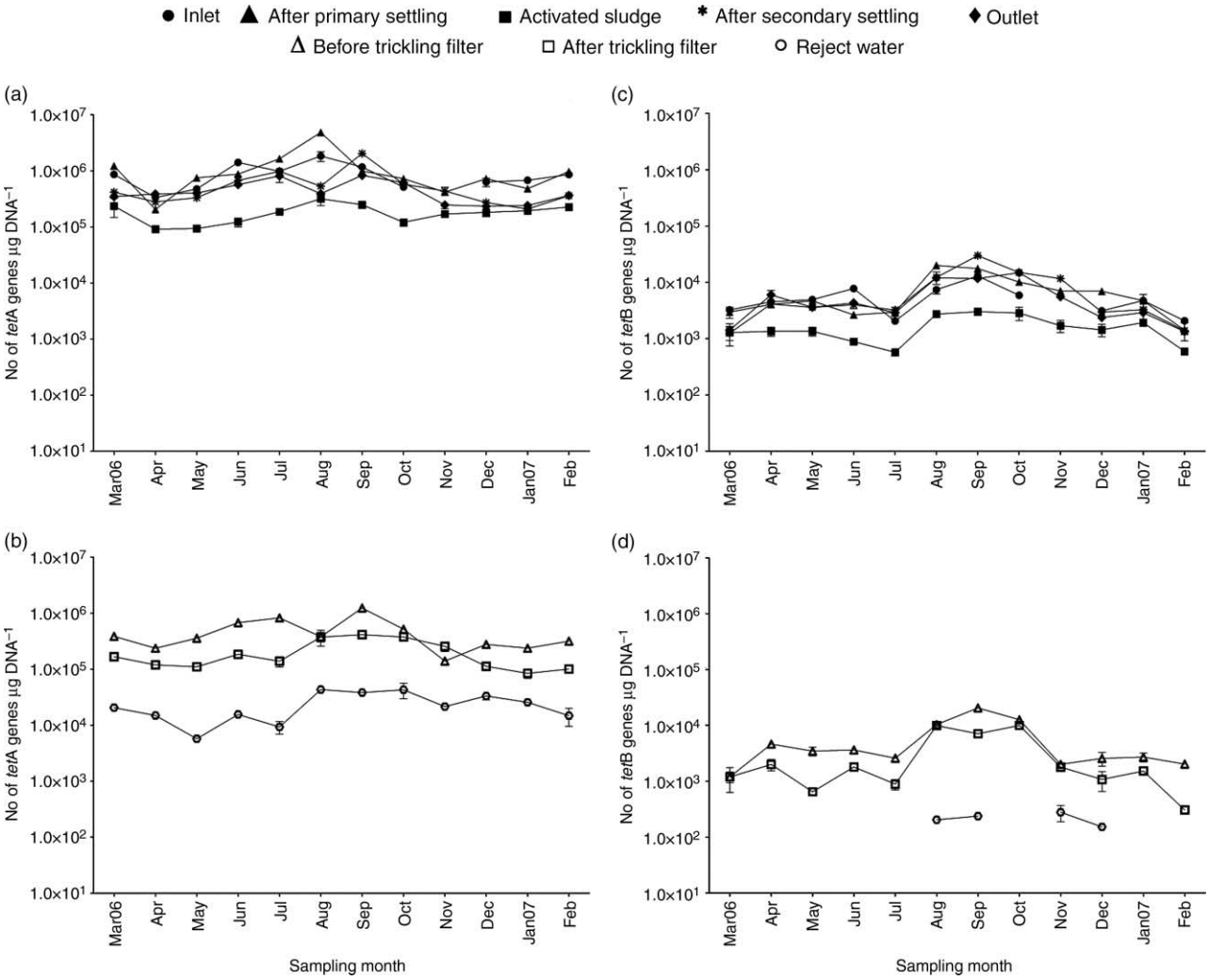

Figure 3 | Concentrations of the *tet*-genes per µg total DNA, at Ryaverket, determined using LUX™ real-time PCR, in grab-samples taken monthly from March 2006 to February 2007. (a-b) *tet*A; (c-d) *tet*B. Error bars show standard deviation for the triplicate measurements in the real-time PCR assay.

coliform bacteria. The reduction of total coliform bacteria from April 2006 to February 2007 was in the same range, 0.8–2.1 $\log_{10}$ units, as the reduction of *tet*A from 0.6 to 2.5, and *tet*B, from 0.5 to 1.6.

As is the purpose of the WWTP, COD, solids, N_{tot}, P_{tot} and faecal bacteria were reduced throughout the WWTP (Figure 4). When *tet*A and *tet*B concentrations were correlated to the physiochemical parameters, a statistically significant correlation was found between reduction of both genes and the reduction of COD, 0.1–0.5 $\log_{10}$ units and P_{tot}, 0.6–1.3 $\log_{10}$ units (Table 3). The reduction of *tet*A and *tet*B was also significantly correlated with reduction of biomass, 0.4–1.8 $\log_{10}$ units.

DISCUSSION

In this study we found that both the genes *tet*A and *tet*B occur throughout the year, and throughout the entire treatment process in a full-scale WWTP (Figures 2 and 3). A reduction of *tet*A and *tet*B concentrations is observed over the treatment process, and the reduction may partly be explained by precipitation and sedimentation in the WWTP. This is supported by a significant correlation in reduction of *tet*A and *tet*B, respectively, related to water volume, to reduction of biomass, COD and P_{tot} (Table 3). The reasons for P_{tot} removal are precipitation and sedimentation, and COD is reduced through biological

Table 2 | Correlation of *tet*A and *tet* B gene per 100 ml wastewater, to biomass (dsDNA) and faecal indicator Bacteria (coliform bacteria and *E. coli*) for each of the eight sites

	Inlet (Site 1)				**After primary settling (Site 2)**			
	***tet*A**	***tet* B**	**Coliform**	***E. coli***	***tet*A**	***tet* B**	**Coliform**	***E. coli***
*tet*A								
tet B	0.500				0.294			
Coliform	**0.833***	**0.685**			**0.818**	0.436		
E. coli	0.517	0.614	**0.697**		**0.891**	0.327	**0.900**	
dsDNA	**0.836**	0.564	0.518	0.210	0.448	0.007	0.082	0.173
	Activated sludge (Site 3)				**After secondary settling (Site 4)**			
	***tet*A**	***tet* B**	**Coliform**	***E. coli***	***tet*A**	***tet* B**	**Coliform**	***E. coli***
*tet*A								
tet B	0.252				**0.692**			
Coliform	0.141	0.059			**0.657**	0.456		
E. coli	−0.045	−0.327	**0.615**		**0.745**	0.612	**0.869**	
dsDNA	**0.804**	0.287	0.282	−0.009	**0.678**	**0.678**	0.067	0.176
	Outlet (Site 5)				**Before trickling filter (Site 6)**			
	***tet*A**	***tet* B**	**Coliform**	***E. coli***	***tet*A**	***tet* B**	**Coliform**	***E. coli***
*tet*A								
tet B	**0.790**				**0.841**			
Coliform	**0.645**	**0.773**			**0.664**	0.569		
E. coli	**0.618**	**0.609**	**0.745**		**0.667**	**0.673**	**0.895**	
dsDNA	**0.881**	**0.636**	0.309	0.373	**0.811**	**0.781**	0.155	0.288
	After trickling filter (Site 7)				**Reject water (Site 8)**			
	***tet*A**	***tet* B**	**Coliform**	***E. coli***	***tet*A**	***tet* B**	**Coliform**	***E. coli***
*tet*A								
tet B	**0.601**				**1.000**			
Coliform	0.027	0.391			0.429	−0.400		
E. coli	0.545	0.455	0.409		0.425	0.211	**0.885**	
dsDNA	**0.720**	0.399	0.255	0.136	0.343	0.400	0.320	0.091

*Bold numbers denotes statistical significant correlations, $p < 0.05$ (Spearmans coefficient two variance analysis).

treatment and sedimentation, while biomass is reduced both through sedimentation and biological treatment. Earlier studies have shown that the sedimentation process has an essential role in the removal of bacteria (George *et al.* 2002; Zhang & Farahbakhsh 2007), and the *tet*A and *tet* B reduction are correlated with reduction of total coliform bacteria (Table 3). In addition, the retention time probably plays an important role in reducing both genes, since the reduction of coliform bacteria likely is dependent on retention time (George *et al.* 2002). However, the concentration of *tet*A and *tet* B in relation to total-DNA showed a decrease over the treatment process in most months (Figure 3), indicating that there may be additional mechanisms behind the reduction. The biological processes in the WWTP, i.e. activated sludge, trickling filters and the anaerobic digesters, may have a role in the reduction of the *tet*-genes, because when related to total-DNA, *tet*A and *tet* B levels are lower in or after these processes (Figure 3). In the biological processes, bacteria are utilised for removal of nutrients and organic compounds (Wagner & Loy 2002), and a possible explanation for the reduction may be that bacteria harbouring *tet*A and *tet* B are not favoured in these processes. Only part of the wastewater is treated in the trickling filters, and the treated water is pumped back into

Table 3 | Correlation of reduction (Inlet compared to Outlet) of number of *tet*A and *tet*B genes per 100 ml wastewater, to reduction of biomass (dsDNA), faecal bacteria (coliform bacteria and *E. coli*) and physiochemical parameters

	*tet*A	*tet*B	Coliform	dsDNA	Solids	COD	P_{tot}	N_{tot}
*tet*A								
*tet*B	**0.918***							
Coliform	**1.000**	**0.891**						
dsDNA	**0.873**	**0.891**	**0.873**					
Solids	0.533	0.465	0.533	**0.653**				
COD	**0.727**	**0.718**	0.564	**0.601**	0.544			
P_{tot}	**0.791**	**0.882**	0.473	**0.580**	0.463	**0.762**		
N_{tot}	**0.815**	**0.856**	0.533	**0.662**	**0.580**	**0.792**	**0.925**	

*Bold numbers denotes statistical significant correlation, $p < 0.05$ (Spearmans coefficient two variance analysis).

the activated sludge system. As a consequence of this, the reducing effect of the trickling filter on *tet*-genes is lost, but in a system where all wastewater passes through nitrifying trickling filters removal of *tet*-genes would be significant. The fact that the concentrations of both genes in relation to total DNA are significantly lower in the reject water compared to all other sites in the WWTP (Table 1, Figure 3) indicates that the 20–30 days of anaerobic digestion at 37°C play a major role in reducing *tet*-genes. That the WWTPs in fact reduce concentration of *tet*-genes are supported by an earlier study (Auerbach *et al.* 2007), and this study suggested that lowered *tet*-gene abundance was primarily attributed to lowered amount of biomass. However, it did not offer any further explanation for the reduction or mechanisms behind the reduction.

The higher concentrations of *tet*A and *tet*B, related to volume, in sampling sites with high biomass concentrations, suggest that the amount of biomass may influence the total number of *tet*A and *tet*B genes. Our finding that *tet*A concentrations significantly correlated, in most of the sampling sites, to higher total biomass, and that *tet*A and *tet*B reductions correlated with total DNA reduction, further support this. Furthermore, when the genes are related to total DNA generally no reduction in primary settling compared to inlet is detected (Table 1, Figure 3), suggesting that the removal of the genes is related to general removal of organic matter in the primary settlers. In addition, an earlier study found that the number of tetracycline-resistant bacteria in activated sludge increased with increasing amount of biomass (Kim *et al.* 2007*a*,*b*).

It could be expected that *tet*B generally would exhibit lower concentrations than *tet*A, because *tet*A is reported to exhibit higher concentration in different types of wastewater environments than *tet*B (Börjesson *et al.* 2009*a*). High concentrations of *tet*A and *tet*B might also be expected in WWTPs, because both genes are commonly found in a wide range of gram-negative bacteria (Chopra & Roberts 2001), carried on easily transferable plasmids, which are common both in human colon and in wastewater (Penders *et al.* 2007; Reinthaler *et al.* 2003). If concentration of *tet*A and *tet*B is connected to concentration of faecal bacteria, it could be expected that gene concentration would correlate to faecal bacteria concentration. This was the case for the *tet*A related to water volume, but not for *tet*B (Table 2). Other studies have shown that *tet*A is

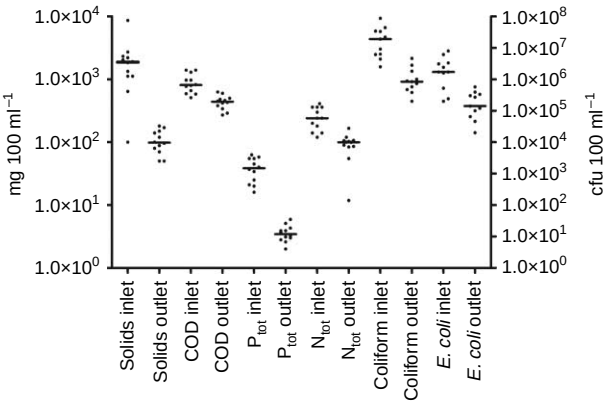

Figure 4 | Concentrations of faecal bacteria, coliform bacteria and *E. coli* (cfu 100 ml^{-1}) and physiochemical parameters (mg 100 ml^{-1}), in *inlet* and *outlet* samples taken monthly from April 2006 to February 2007 at Ryaverket. ●denotes the concentration for each month and the horizontal lines indicate the median of concentrations over the year for the respective site.

more frequently detected than *tet* B in wastewater and soil over time (Guillaume *et al.* 2000; Auerbach *et al.* 2007; Patterson *et al.* 2007; Börjesson *et al.* 2009*a*). Taken together, this may indicate that tetracycline-resistant bacteria and *tet*A are common in environments connected to wastewater, and *tet*A may be more prevalent in bacterial populations than *tet* B.

CONCLUSIONS

The current study describes that *tet*A and *tet* B gene concentrations have a stable occurrence over time in an activated sludge and trickling filter municipal WWTP, using our recently developed quantitative real-time PCR assays. Furthermore, the gene concentration decreases during the treatment process. Earlier studies have described similar results of *tet*-genes in WWTPs, but have neither performed such a long-term investigation nor offered an explanation to the removal. We show that the reduction likely is connected to removal of bacteria, biomass and solid material in the treatment process, implying that precipitation and sedimentation processes play a major role. In addition, biological processes appear to contribute to reducing the abundance of the genes. The number of *tet*A and *tet* B genes in the WWTP seems also to be influenced by the amount of biomass. Although, the concentrations are reduced, there are still high concentrations in the outlet, implicating WWTPs as a potential source for dissemination of tetracycline resistance.

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REFERENCES

Auerbach, E. A., Seyfried, E. E. & McMahon, K. D. 2007 Tetracycline resistance genes in activated sludge wastewater treatment plants. *Water Res.* **41**(5), 1143–1151.

Börjesson, S., Dienus, O., Jarnheimer, P. A., Olsen, B., Matussek, A. & Lindgren, P. E. 2009*a* Quantification of genes encoding resistance to aminoglycosides, ß-lactams and tetracyclines in wastewater environments by real-time PCR. *Int. J. Environ. Health. Res.* **19**(3), 219–230.

Börjesson, S., Melin, S., Matussek, A. & Lindgren, P. E. 2009*b* A seasonal study of the *mec*A gene and *Staphylococcus aureus* including methicillin-resistant *S. aureus* in a municipal wastewater treatment plant. *Water Res.* **43**(4), 925–932.

Casas, C., Andersson, E. C., Ojo, K. K., Keith, I., Whelan, D., Rainnie, D. & Roberts, M. 2004 Characterization of pRAS1-like plasmids from atypical North American psychrophilic *Aeromonas salmonicida*. *FEMS Microbial. Lett.* **242**(1), 59–63.

Chopra, I. & Roberts, M. 2001 Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiol. Mol. Biol. Rev.* **65**(2), 232–260.

George, I., Crop, P. & Servais, P. 2002 Fecal coliform removal in wastewater treatment plants studied by plate counts and enzymatic methods. *Water Res.* **36**(10), 2607–2617.

Guillaume, G., Verbrugge, D., Chasseur-Libotte, M., Moens, W. & Collard, J. 2000 PCR typing of tetracycline resistance determinants (*Tet* A-E) in *Salmonella enterica* serotype Hadar and in the microbial community of activated sludges from hospital and urban wastewater treatment facilities in Belgium. *FEMS Microbiol. Ecol.* **32**(1), 77–85.

Karthikeyan, K. G. & Meyer, M. T. 2006 Occurrence of antibiotics in wastewater treatment facilities in Wisconsin, USA. *Sci. Total Environ.* **361**(1–3), 196–207.

Kim, S., Jensen, J. N., Aga, D. S. & Weber, A. S. 2007 Tetracycline as a selector for resistant bacteria in activated sludge. *Chemosphere* **66**(9), 1643–1651.

Kim, S., Jensen, J. N., Aga, D. S. & Weber, A. S. 2007 Fate of tetracycline resistant bacteria as a function of activated sludge process organic load and growth rate. *Water Sci. Technol.* **55**(1–2), 291–297.

Patterson, A. J., Colangeli, R., Spigaglia, P. & Scott, K. P. 2007 Distribution of specific tetracycline and erythromycin resistance genes in environmental samples assessed by macroarray detection. *Environ. Microbiol.* **9**(3), 703–715.

Peak, N., Knapp, C. W., Yang, R. K., Hanfelt, M. M., Smith, M. S., Aga, D. S. & Graham, D. W. 2007 Abundance of six tetracycline resistance genes in wastewater lagoons at cattle feedlots with different antibiotic use strategies. *Environ. Microbiol.* **9**(1), 143–151.

Penders, J., Thijs, C., Brandt, P. A., Kummeling, I., Snijders, B., Stelma, F., Adams, H., van Ree, R. & Stobberingh, E. E. 2007 Gut microbiota composition and development of atopic manifestations in infancy: the KOALA birth cohort study. *Gut* **56**(5), 661–667.

Reinthaler, F. F., Posch, J., Feierl, G., Wust, G., Haas, D., Ruckenbauer, G., Mascher, F. & Marth, E. 2003 Antibiotic resistance of *E. coli* in sewage and sludge. *Water Res.* **37**(8), 1685–1690.

Rhodes, G., Huys, G., Swings, J., McGann, P., Hiney, M., Smith, P. & Pickup, R. W. 2000 Distribution of oxytetracycline resistance plasmids between aeromonads in hospital and aquaculture environments: implication of Tn1721 in dissemination of the tetracycline resistance determinant *tet* A. *Appl. Environ. Microbiol.* **66**(9), 3883–3890.

Roberts, M. C. 2003 Tetracycline therapy: update. *Clin. Infect. Dis.* **36**(4), 462–467.

Roberts, M. C. 2005 Update on acquired tetracycline resistance genes. *FEMS Microbiol. Lett.* **245**(2), 195–203.

Wagner, M. & Loy, A. 2002 Bacterial community composition and function in sewage treatment systems. *Curr. Opin. Biotechnol.* **13**(3), 218–227.

Zhang, K. & Farahbakhsh, K. 2007 Removal of native coliphages and coliform bacteria from municipal wastewater by various wastewater treatment processes: implications to water reuse. *Water Res.* **41**(12), 2816–2824.

Zwenger, S. R. & Gillock, E. T. 2009 Bacteria isolated from sewage influent resistant to ciprofloxacin, chloramphenicol and tetracycline. *J. Environ. Sci. Health A. Tox. Hazard Subst. Environ. Eng.* **44**(2), 123–129.

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