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Propensity of activated sludge to amplify or attenuate tetracycline resistance genes and tetracycline resistant bacteria: A mathematical modeling approach

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ABSTRACT

The overall goal of this study was to quantify the propensity of the activated sludge (AS) process at three wastewater treatment plants (WWTP) to amplify or attenuate tetracycline resistant bacteria (TRB) and tetracycline resistance genes (TRG). Accordingly, the abundance and fraction of TRB and seven TRG in different unit operations of these WWTP were analytically measured and modeled using a mass balance approach widely used for AS design. Based on the model, the AS process of the different WWTP neither amplified nor attenuated the TRB and TRG fractions. Of the TRG tested, the ribosomal protection genes, *tet*(O) and *tet*(W) were the most abundant, along the treatment train of the WWTP, on all sampling dates and sampling locations. Significant amounts of TRB and TRG were discharged in the effluent streams. Notably, in selected samples, the fraction of TRB increased in response to ultraviolet disinfection of treated wastewater compared to chlorination. This study therefore implicates wastewater treatment processes as significant point sources of tetracycline resistance determinants to the environment, and provides a mathematical basis to compute the production capacity of these determinants in the AS process.

1. Introduction

The proliferation of antibiotic resistant bacteria and antibiotic resistance genes in the environment is portending to be a major public-health crisis (Levy, 2002; Baquero et al., 2008). Based on previous efforts, which have centered on independently characterizing either the abundance of antibiotic resistant bacteria (Guardabassi et al., 2002; Reinthaler et al., 2003) or antibiotic resistance genes (Szczepanowski et al., 2004; Auerbach et al., 2007), wastewater treatment plants (WWTP) have been implicated as major contributors of antibiotic resistant bacteria and antibiotic resistance genes to receiving water bodies.

The possibility of selection for antibiotic resistance determinants in WWTP conceptually exists. The influent streams of WWTP usually contain heavy metals, anti-microbial agents, detergents and quaternary ammonium compounds, all of which have been linked to co- or cross-resistance to antibiotics (Silver and Phung, 1996; Alonso et al., 2001; Levy, 2002; Nies, 2003; Ruiz et al., 2003; Stepanauskas et al., 2005; Baker-Austin et al., 2006). However, little quantitative or conclusive evidence for such selection has been found. Further, the impact of activated sludge (AS) operating conditions on the production of antibiotic resistant bacteria or selective antibiotic resistance genes therein has not yet been described using a quantitative framework. Such a framework is needed, for instance, if future designs of WWTP are considering removal of antibiotic resistant bacteria and resistance genes. Accordingly, it would be meaningful if mass balance approaches traditionally used for WWTP design (Grady et al., 1999) could be adapted to describe the fate of antibiotic resistant bacteria and resistance genes in them.

The overall goal of this study was to determine possible attenuation or amplification in the abundance of tetracycline resistant bacteria (TRB) and several tetracycline resistance genes (TRG) along the treatment train of three distinctly operated full scale WWTP via a combination of analytical measurements and mathematical modeling approaches. The specific objectives were to: (1) quantify the abundance and fraction of TRB and TRG in different unit operations of three WWTP and (2) determine the TRB and TRG production capacity of the AS process of WWTP using a traditional mass balance approach (Grady et al., 1999).

2. Materials and methods

2.1. Sampling of TRB and TRG at full-scale wastewater treatment plants

Three WWTP (designated A, B, C) located in New York and Connecticut with different modes of operation were studied. Plants A

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Nomenclature

TRB	tetracycline resistant bacteria
TRG	tetracycline resistance genes
WWTP	wastewater treatment plants
BNR	biological nitrogen removal
PE	primary clarifier effluent
AS	activated sludge
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
DNA plasmid conc.	DNA plasmid concentration ($\mu g\ mL^{-1}$)
bp	base pair
X_{TRB}	TRB concentration in AS (mg COD L^{-1})
Θ_c	solids retention time (d)
τ	hydraulic retention time (d)
$X_{TRB,\ in}$	TRB concentrations in influent stream to AS (mg COD L^{-1})
$X_{TRB,\ eff}$	TRB concentrations in secondary clarifier effluent (before disinfection) (mg COD L^{-1})
$Y_{n,\ TRB}$	net yield coefficient of TRB (mg COD mg COD^{-1})
b_{TRB}	decay coefficient of TRB (d^{-1}) = 0.408 d^{-1}, assumed to equal that of heterotrophic bacteria in AS (Grady et al., 1999)
ΔS_{TRB}	COD removal by TRB in AS (mg L^{-1}), cannot be explicitly calculated and therefore lumped with $Y_{n,\ TRB}$ to operationally define production capacity (PC)
X_{TRG}	TRG concentration in AS samples (individual genes or sum thereof, copies L^{-1})
$X_{TRG,\ in}$	TRG concentration in PE (individual genes or sum thereof, copies L^{-1})
$X_{TRG,\ eff}$	TRG concentrations in secondary clarifier effluent (individual genes or sum thereof, copies L^{-1})
b_{TRG}	decay coefficient of TRG (d^{-1}) = 0.408 d^{-1}, assumed to equal that of TRB
PC_{TRB}	production capacity of TRB in AS (mg COD L^{-1})
PC_{TRG}	production capacity of TRG in AS (copies L^{-1})

and C are designed and operated mainly for removal of organic carbon, whereas Plant B is engaged in biological nitrogen removal employing a five-stage Bardenpho configuration. Plant A practices seasonal disinfection during June–October, whereas Plants B and C employ UV irradiation and chlorination, respectively. Therefore samples after disinfection were not available in Plant A during the entire sampling campaign. To be consistent, secondary effluent samples in Plant A were always collected pre-chlorination during the disinfection period. Samples were obtained during the period January–July 2007 at several points in each WWTP including primary effluent (PE), AS and before and after disinfection (for Plants B and C). One litre grab samples were collected in ethanol-sterilized Nalgene HDPE sample bottles and stored immediately on ice. Samples for endpoint and quantitative polymerase chain reaction (PCR) were concentrated by centrifugation (7000 rpm for 5 min) or filtration through 1.2 μm GF/C glass fiber filter (Millipore, Ann Arbor, MI). The resulting cell pellets and filters were stored at −80 °C prior to subsequent molecular analysis.

2.2. Quantification of TRB and TRG

Concentrations of total heterotrophic bacteria and TRB in colony forming units per milliliter (CFU mL^{-1}) were measured by plating in triplicate on R2A agar containing 0 and 32 $\mu g\ mL^{-1}$ tetracycline, respectively. The TRB fraction relative to total heterotrophic bacterial abundance was also computed. Seven TRG [*tet*(A) *tet*(B) *tet*(C) *tet*(M) *tet*(O) *tet*(Q) *tet*(W)], were targeted in this study because of their presence in a wide variety of environments (Roberts, 1996). Of these, *tet*(A), *tet*(B) and *tet*(C) are involved in tetracycline efflux and *tet*(M), *tet*(O), *tet*(Q) and *tet*(W) are involved in ribosomal protection. Samples from February, April, and July 2007 were selected for characterization of TRG abundance at three different seasonal temperatures ranging from 13.2 to 21.7 °C. Following DNA extraction (DNeasy Blood & Tissue kit, Qiagen, Irvine, CA), endpoint PCR was performed to confirm the presence of the seven selected TRG in PE samples before quantification through different unit processes via qPCR. Standard curves for qPCR were constructed via serial decimal dilution of plasmids containing cloned TRG inserts kindly provided by Dr. Ludek Zurek (Kansas State University, Manhattan, KS) and Dr. Anthony Yannarell (University of Illinois, Urbana-Champaign, IL). TRG abundance was determined by qPCR (copies mL^{-1}, Eq. (1)) and normalized to bacterial 16S rRNA gene abundance, quantified using bacterial primers BACT1369F and PROK1492R (Suzuki et al., 2000), to account for variability of total bacterial abundance in samples. Endpoint PCR and qPCR conditions (Table 1) were similar to those employed previously (Suzuki et al., 2000; Aminov et al., 2001; Aminov et al., 2002; Macovei and Zurek, 2006; Auerbach et al., 2007). The concentrations of the seven TRG in each unit process were added to give the 'lumped' TRG concentration and were used to compare the overall fates of the TRG in each of the three WWTP.

$$\text{TRG} - \text{conc.}\ (\text{copies mL}^{-1}) = 6.023 \times 10^{23}\left(\frac{\text{DNA plasmid conc.}\ (\mu g L^{-1})}{(\text{Plasmid} - \text{size}) \times 660\ \text{g bp mole}^{-1}}\right) \quad (1)$$

2.3. Production capacity of TRB and TRG in AS

The concentrations of TRB in CFU mL^{-1} in the primary and secondary effluent and AS samples were converted to mg COD L^{-1} based on an average cell mass of 2.8×10^{-13} g^{-1} cell (Madigan and Martinko, 2006) and correlated to WWTP operating characteristics via a steady-state mass balance equation (Eq. (2), after (Grady et al., 1999)).

$$X_{TRB} = \left(\frac{\theta_C}{\tau}\right)\left[\left(\frac{X_{TRB,in} - X_{TRB,eff}}{1 + b_{TRB}\theta_C}\right) + \left(\frac{Y_{n,TRB}\Delta S_{TRB}}{1 + b_{TRB}\theta_C}\right)\right] \quad (2)$$

The product ($Y_{n,TRB}$ ΔS_{TRB}) was operationally defined as the net production capacity of AS and physically signifies the amount of TRB biomass produced therein. The definition of production capacity in this manner also obviates the calculation of carbonaceous substrate degradation attributed specifically to TRB, which cannot be done with certainty. The TRB production capacity (PC_{TRB}) was calculated for each sampling date at the three WWTP via Eq. (3), obtained by re-arranging Eq. (2).

$$PC_{TRB} = Y_{n,TRB}\Delta S_{TRB} = \left[\frac{X_{TRB}\tau}{\theta_C}(1 + b_{TRB}\theta_C) - X_{TRB,in} + X_{TRB,eff}\right] \quad (3)$$

By analogy, a similar production capacity for TRG (PC_{TRG}) can be formulated, (Eq. (4)).

$$PC_{TRG} = \left[\frac{X_{TRG}\tau}{\theta_C}(1 + b_{TRG}\theta_C) - X_{TRG,in} + X_{TRG,eff}\right] \quad (4)$$

Table 1
Primers for PCR and qPCR assays targeting TRG.

Primer	Primer sequence	Annealing temperature (°C)	PCR assay	References
tet(A)f	GCTACATCCTGCTTGCCTTC	55	PCR and qPCR	Macovei and Zurek (2006)
tet(A)r	CATAGATCGCCGTGAAGAGG			
tet(B)f	TTGGTTAGGGGCAAGTTTTG	55	PCR	Auerbach et al. (2007)
tet(B)r	GTAATGGGCCAATAACACCG			
tet(B)fq	TACGTGAATTTATTGCTTCGG	61	qPCR	Aminov et al. (2002)
tet(B)rq	ATACAGCATCCAAAGCGCAC			
tet(C)f	CTTGAGAGCCTTCAACCCAG	55	PCR and qPCR	Ng et al. (2001))
tet(C)r	ATGGTCGTCATCTACCTGCC			
tet(M)f	AGTTTTAGCTCATGTTGATG	55	PCR	Doherty et al. (2000)
tet(M)r	TCCGACTATTTGGACGACGG			
tet(M)fq	GTGGACAAAGGTACAACGAG	55	qPCR	Ng et al. (2001)
tet(M)rq	CGGTAAAGTTCGTCACACAC			
tet(O)f	AACTTAGGCATTCTGGCTCAC	55	PCR and qPCR	Macovei and Zurek (2006)
tet(O)r	TCCCACTGTTCCATATCGTCA			
tet(Q)f	TTATACTTCCTCCGGCATCG	55	PCR	Ng et al. (2001)
tet(Q)r	ATCGGTTCGAGAATGTCCAC			
tet(Q)fq	AGAATCTGCTGTTTGCCAGTG	63	qPCR	Aminov et al. (2001)
tet(Q)rq	CGGAGTGTCAATGATATTGCA			
tet(W)f	GAGAGCCTGCTATATGCCAGC	64	PCR and qPCR	Aminov et al. (2001)
tet(W)r	GGGCGTATCCACAATGTTAAC			
BACT1369F	CGGTGAATACGTTCYCGG	55	qPCR	Suzuki et al. (2000)
PROK1492R	GGWTACCTTGTTACGACTT			

3. Results

3.1. TRB concentrations and fractions in WWTP unit processes

The concentrations of TRB in PE samples at the three WWTP ranged from 1.6×10^4 to 1.7×10^5 CFU mL^{-1} (Fig. 1) and were comparable to TRB concentrations measured in the PE in other studies (Guardabassi et al., 2002; Kim et al., 2007). The TRB concentrations in AS samples were statistically similar to TRB concentrations in the PE ($p > 0.05$) at Plants A and B, but moderately higher and statistically different at Plant C ($p = 0.003$, Fig. 1). The TRB concentrations measured downstream of AS were significantly lower primarily due to secondary clarification. Despite these differences in the measured TRB *concentrations*, the TRB *fractions* in all unit processes at all WWTP were statistically similar ($p > 0.05$, Fig. 2). A pair-wise comparison revealed that the TRB fractions in Plant B after UV disinfection were statistically higher than before disinfection on three of the six sampling dates (Fig. 2b). However, due to significant day-to-day variability in the specific values of the measured TRB fractions, when considered together, the average TRB fractions before and after UV disinfection were not significantly different at the 95% confidence level ($p = 0.19$). The increase in TRB fraction was singular to UV disinfection and was never observed with chlorination (Fig. 2c). There were no systematic seasonal trends in the TRB concentrations or fractions during this study.

3.2. TRG concentrations and fractions in WWTP unit processes

All seven TRG were detected in PE samples, based on an initial screening via endpoint PCR (data not shown). The lumped TRG concentrations in the PE and AS samples were statistically similar ($0.20 < p < 0.28$) at all WWTP and significantly declined downstream of the AS process (Fig. 3). However, the lumped TRG *fraction* at the different unit processes was largely similar in all WWTP (Fig. 4), In pair-wise comparisons of the TRG fraction in each downstream process with the PE samples, only the AS process in Plant A was statistically different ($p = 0.003$). Considered individually, *tet*(O), *tet*(Q), and *tet*(W) were generally the most abundant TRG in several WWTP unit processes (data subset shown in Fig. 5). The fractions of *tet*(O), *tet*(Q) and *tet*(W) were also in general higher than those of *tet*(A), *tet*(B), *tet*(C) and *tet*(M) (data not shown). Consistent trends as a function of seasonal temperature in TRG concentrations or fractions could not be inferred (Figs. 3 and 4). Systematic trends in the TRG fractions were also not observed through different unit processes of the treatment train at any of the three WWTP (data not shown).

3.3. Production capacity of TRB and TRG in AS

Despite the different modes of operation at the three WWTP studied, the net production capacity of TRB in their respective AS reactors was statistically similar ($p > 0.05$, Table 2a) and not different from zero. The production capacity of lumped TRG in Plants A and C was negative, suggesting a moderate degree of attenuation. The lumped TRG production capacity in Plant B was not statistically different from zero ($p = 0.29$). The individual production capacities of *tet*(O), *tet*(Q) and *tet*(W) showed statistically significant attenuation (negative values) (Table 2b). The production capacities for the other genes were in general not statistically significantly different from zero (Table 2b).

4. Discussion

4.1. Production capacities of TRB and TRG in AS reactors

AS bioreactors in WWTP are primarily designed to maximize biological substrate removal by promoting factors for fostering bacterial retention and growth. Additionally, influent streams of WWTP also contain several compounds that could lead to co-resistance and cross-resistance towards antibiotics including tetracycline (Silver and Phung, 1996; Alonso et al., 2001; Levy, 2002; Nies, 2003; Ruiz et al., 2003; Stepanauskas et al., 2005; Baker-Austin et al., 2006). These include chemicals present in several domestic and industrial use products such as heavy metals, detergents, microbial antifouling agents and constituents of personal care products, for instance quaternary ammonium compounds. However, there are several conflicting reports on the amplification (Andersen, 1993; Guardabassi et al., 1998), attenuation (Guardabassi et al., 2002) or lack thereof (Guardabassi et al., 2002) relating to antibiotic resistant bacteria in WWTP. The essentially zero production capacity values determined in this study quantitatively

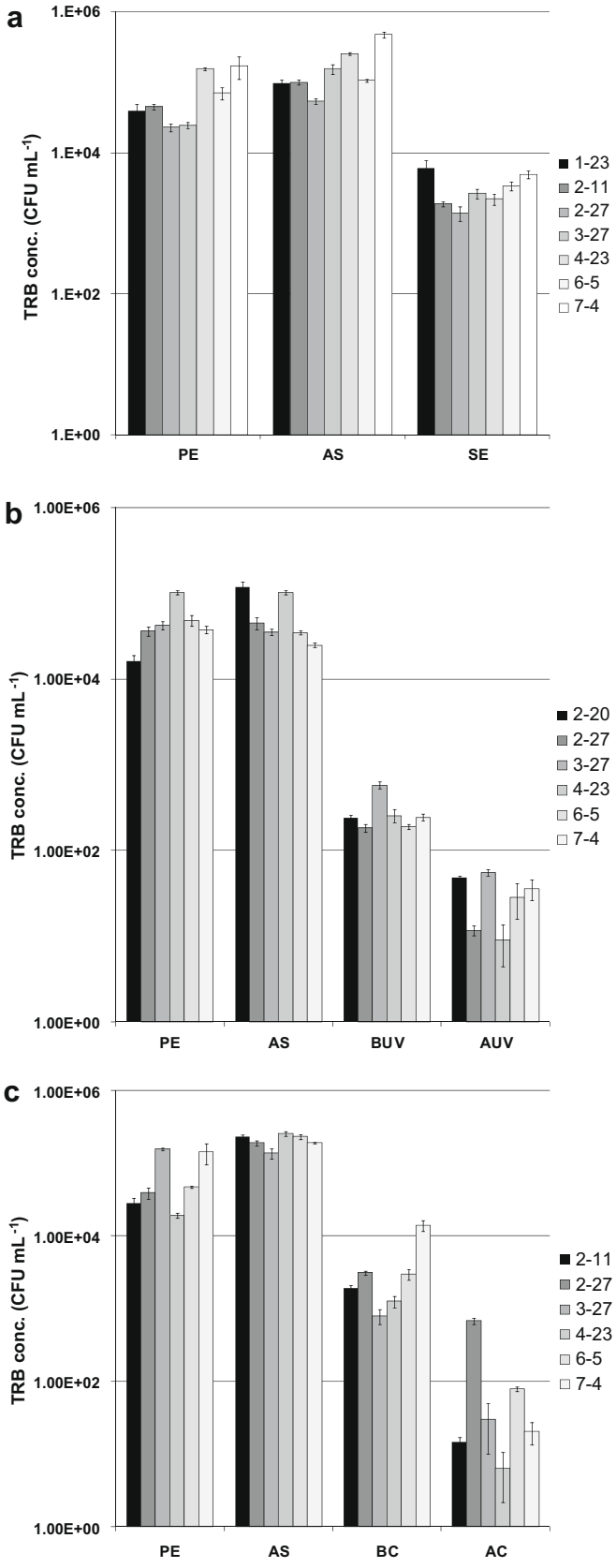

Fig. 1. TRB concentrations at different unit processes in Plant A (a), Plant B (b) and Plant C (c). Unit processes include primary effluent (PE), activated sludge (AS), secondary effluent (SE), before chlorination (BC), before ultraviolet irradiation (BUV), after chlorination (AC) and after UV irradiation (AUV). Error bars represent standard deviation of triplicate plate counts.

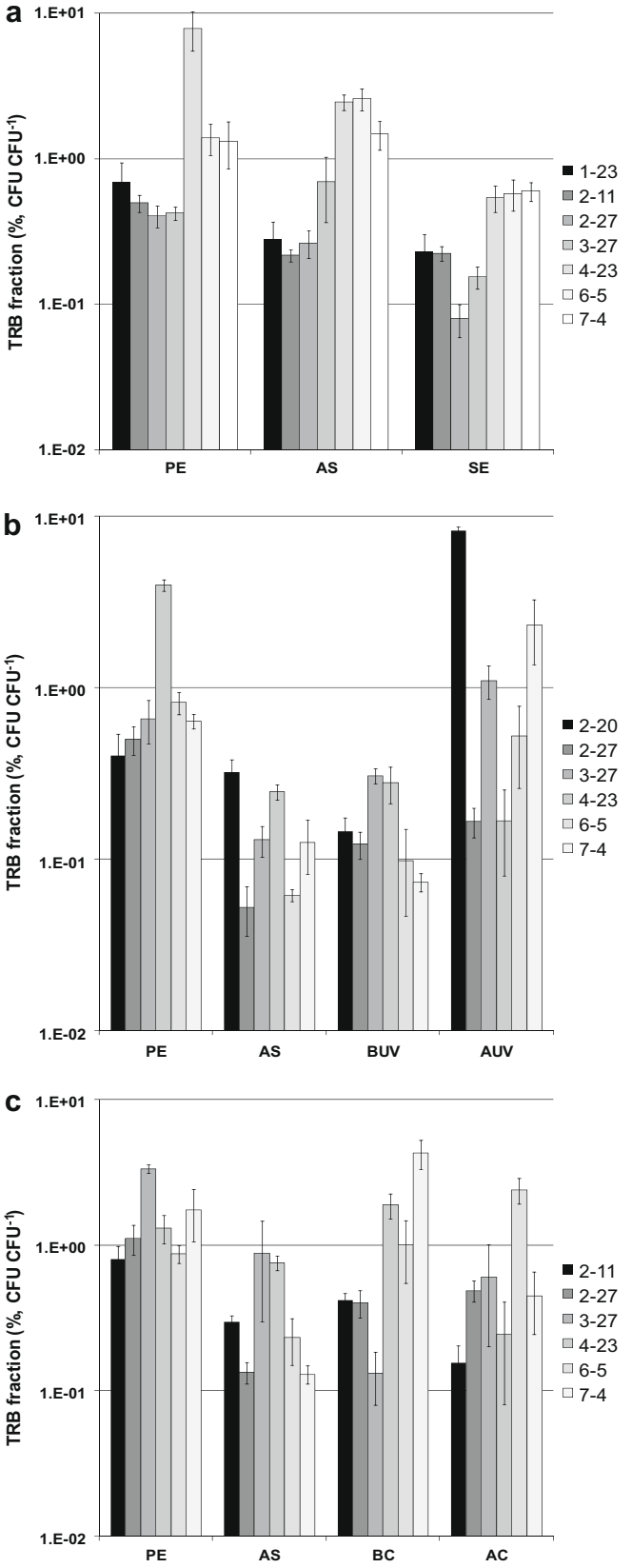

Fig. 2. TRB fractions at different unit processes in Plant A (a), Plant B (b) and Plant C (c). Unit processes follow same nomenclature as Fig. 1. Error bars represent standard deviation of triplicate plate counts.

showed that the AS process did not promote selective enrichment in TRB or TRG fractions, irrespective of the operating mode of the

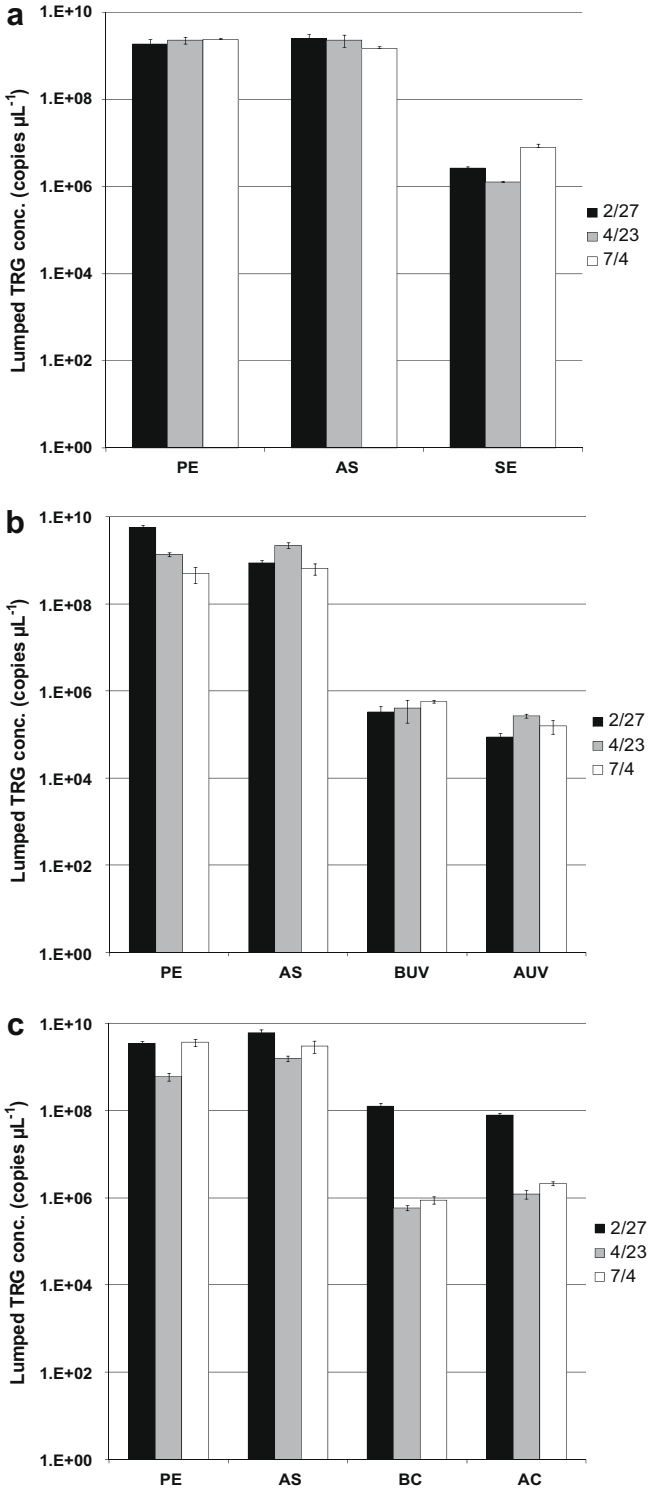

Fig. 3. Lumped TRG concentrations at different unit processes in Plant A (a), Plant B (b) and Plant C (c). Unit processes follow same nomenclature as Fig. 1. Error bars represent standard deviation of triplicate qPCR measurements.

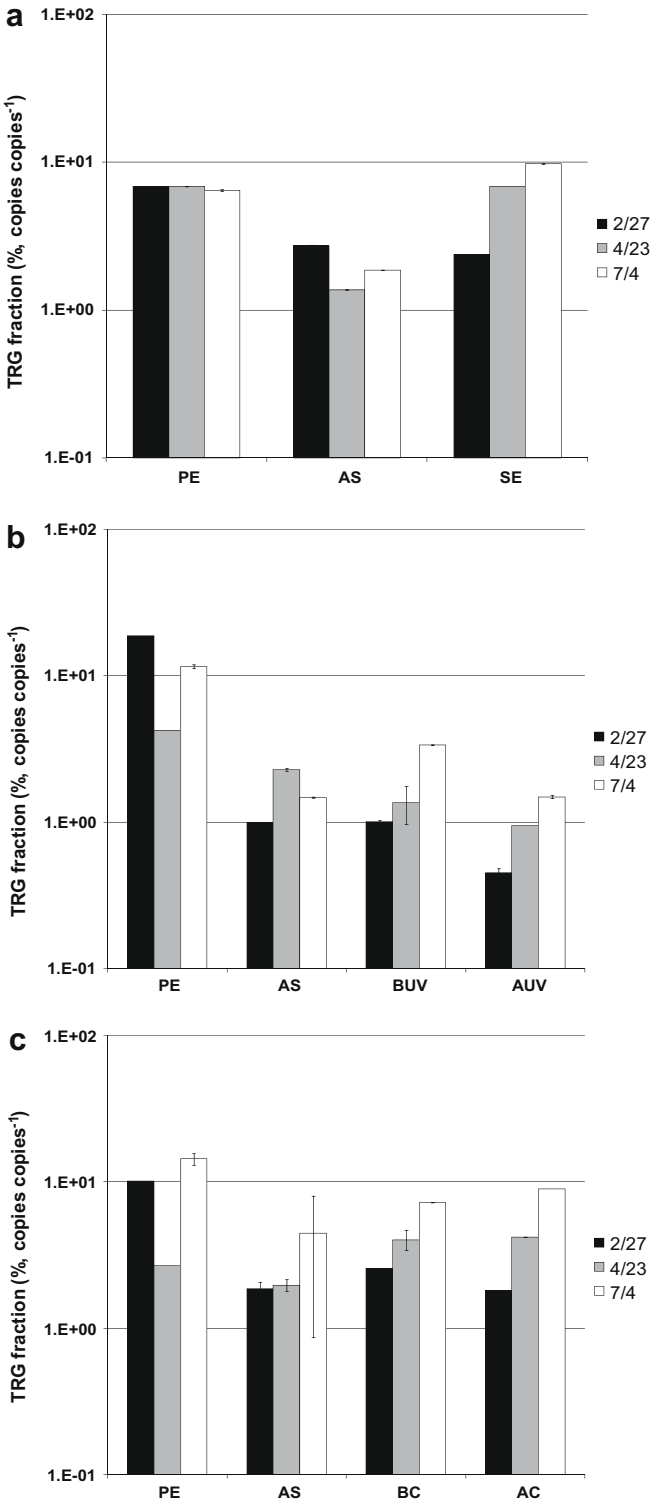

Fig. 4. Lumped TRG fractions (normalized to 16S rRNA gene abundance) at different unit processes in Plant A (a), Plant B (b) and Plant C (c). Unit processes follow same nomenclature as Fig. 1. Error bars represent standard deviation of triplicate qPCR measurements.

three WWTP (Table 2a and b). It should be noted that the mass balance approach assumes that all TRB in AS are heterotrophic in nature and can be described by single values of the biomass decay coefficient (0.408 d^{-1} (Grady et al., 1999)) and net yield coefficients (Eqs. (3) and (4)). Clearly, as more specific characterization of TRB and TRG in AS is conducted, it might be possible to determine TRB and TRG specific coefficients.

4.2. Relative distribution of tetracycline resistance genes in WWTP

Apart from a recent study (Auerbach et al., 2007), the impact of different wastewater treatment unit operations on the abundance of antibiotic resistance genes therein has not been investigated. The previous study focused on quantitative tracking of two TRG,

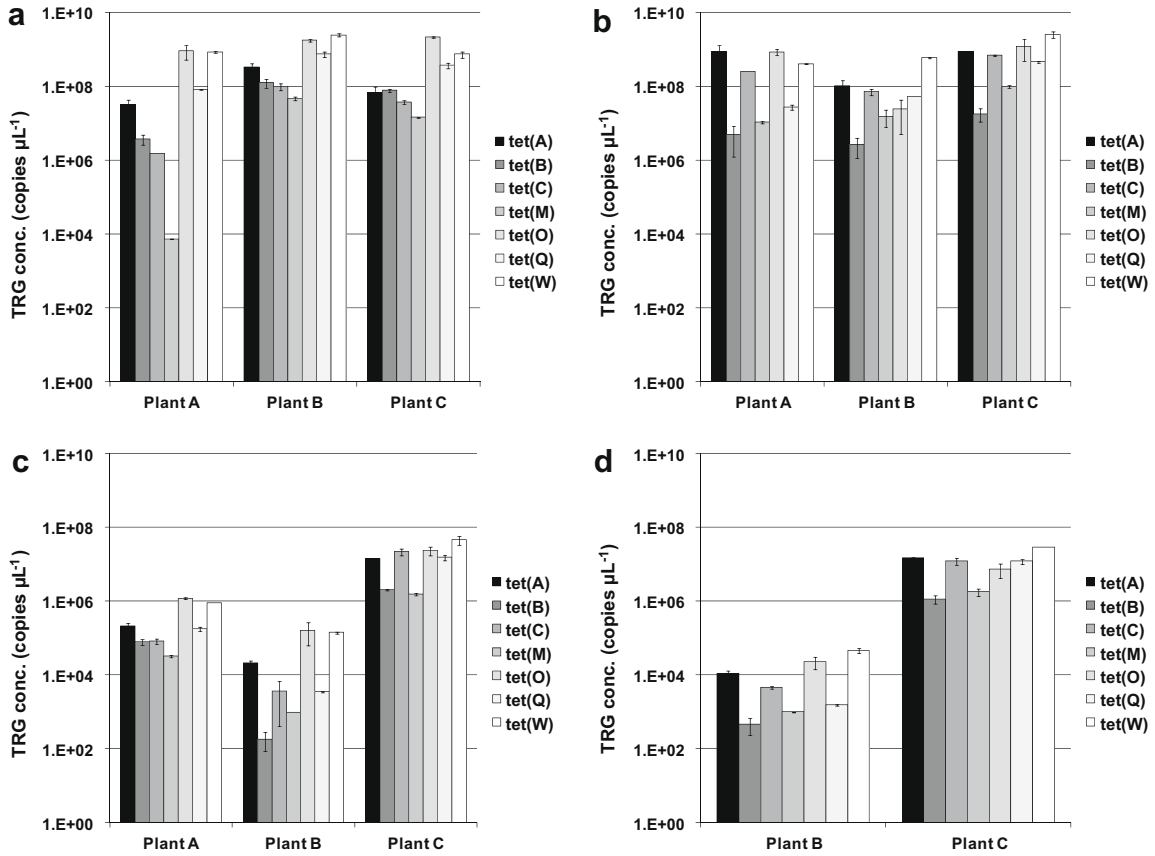

Fig. 5. Representative distribution of individual TRG concentrations as measured on 2/27/2007 three WWTP in PE (a), AS (b), secondary effluent in Plant A or before disinfection in Plants B and C (c) and after disinfection in only plants B and C (d). Error bars represent standard deviation of triplicate qPCR measurements.

Table 2
Production capacities of [a] TRB and lumped TRG, and [b] individual TRG in the AS process at WWTP studied [shaded boxes represent values significantly different from zero, average ± standard deviation reported to two significant figures].

[a] Plant	SRT (d)	HRT (h)	MLSS ($g\ L^{-1}$)	Flow rate ($m^3\ d^{-1}$) × 10^4	Influent BOD ($mg\ L^{-1}$)	TRB production capacity in AS ($mg\ X_{TRB}\ COD\ L^{-1}$)	n_{TRB}	TRG production capacity in AS(TRG copies L^{-1} of AS) × 10^{-12}	n_{TRG}
A	1.7 ± 0.4	3.4 ± 0.6	1.2 ± 0.3	2.1 ± 0.43	122 ± 21	−0.018 ± 0.018	7	−1.9 ± 0.3	3
B	23.1 ± 5.6	6.8 ± 1.5	2.6 ± 0.3	6.8 ± 1.6	115 ± 24	−0.016 ± 0.012	6	−2.3 ± 2.8	3
C	2.0 ± 0.8	2.4 ± 0.4	1.9 ± 0.7	36.±7.3	90 ± 37	−0.017 ± 0.023	7	−2.2 ± 1.5	3

[b] Plant	TRG production capacity (copies TRG L^{-1}) × 10^{12}						
	tet(A)	*tet*(B)	*tet*(C)	*tet*(M)	*tet*(O)	*tet*(Q)	*tet*(W)
A	−0.03 ± 0.14	−0.019 ± 0.021	−0.035 ± 0.053	−0.016 ± 0.017	−1.0 ± 0.26	−0.14 ± 0.10	−0.65 ± 0.16
B	−0.16 ± 0.15	−0.054 ± 0.063	−0.045 ± 0.039	−0.020 ± 0.022	−0.60 ± 1.0	−0.41 ± 0.30	−1.0 ± 1.2
C	−0.24 ± 0.41	−0.063 ± 0.039	−0.060 ± 0.12	−0.010 ± 0.009	−1.0 ± 1.0	−0.22 ± 0.09	−0.62 ± 0.44

tet(G) and *tet*(Q) (Auerbach et al., 2007). In this present study, the set of TRG quantified was expanded. Of the seven TRG probed for in this study, the higher abundance of *tet*(O), *tet*(W) and to an extent, *tet*(Q), which was observed at the three WWTP, is consistent with those in other studies (Scott et al., 2000; Aminov et al., 2001; Billington et al., 2002; Patterson et al., 2007). Specifically, *Bacteroides* spp., which constitute up to 25% of the human intestinal microflora have been shown to act as selective and significant reservoirs (80% of *Bacteroides* spp. isolates) of *tet*(Q) genes (Salyers et al., 2004). *tet*(M), *tet*(O) and *tet*(Q) have also been implicated as the most frequently detected TRG in the human oral microflora (Diaz-Torres et al., 2006). Although not the most abundant TRG in this study, *tet*(M) is the most widespread class of TRG associated with ribosomal protection and is found on conjugative genetic elements belonging to the *Tn916* family (Rice, 1998). *tet*(W) is also especially prevalent in environmental samples (Scott et al., 2000; Billington et al., 2002; Storteboom et al., 2007; Engemann et al., 2008) and has the second most widespread host range known to date. On the other hand, the relatively lower host range of *tet*(A)-(D) (Chopra and Roberts, 2001) could have resulted in their relatively lower concentrations within the WWTP measured in this study (Fig. 5).

4.3. Impact of wastewater disinfection strategies on TRB fraction

UV disinfection is increasingly being practiced in wastewater and drinking water utilities in order to minimize the formation of potentially carcinogenic disinfection byproducts that are associated with chlorination (Bellar et al., 1974). However, based on this

study, UV disinfection could contribute to an increased TRB fraction in wastewater effluent streams. A similar increase in resistance of total coliforms to tetracycline or chloramphenicol in AS effluent streams upon UV irradiation was reported previously and ascribed to increased expression of the commonly plasmid encoded *R*-factor upon exposure to UV (Meckes, 1982). Given that plasmids can also harbor multiple TRG, co-expression of the *R*-factor and TRG could be a possible explanation for the increase in TRB fraction of the bacteria that survive UV exposure.

5. Conclusions

Using widely followed mass balance equations, the TRB and TRG production capacities of AS at three WWTP were in general similar and close to zero. Of the seven TRG tested, two ribosomal protection genes, *tet*(O) and *tet*(W) were most abundant throughout the treatment train, possibly suggesting that these genes might be selectively proliferating in the environment. Nevertheless, it must be stressed that as in previous studies, WWTP are shown to be significant point sources of TRB and TRG to the receiving environment.

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References

Alonso, A., Sanchez, P., Martinez, J.L., 2001. Environmental selection of antibiotic resistance genes. Minirev. Environ. Microbiol. 3, 1–9.

Aminov, R.I., Chee-Sanford, J.C., Garrigues, N., Teferedegne, B., Krapac, I.J., White, B.A., Mackie, R.I., 2002. Development, validation, and application of PCR primers for detection of tetracycline efflux genes of gram-negative bacteria. Appl. Environ. Microbiol. 68, 1786–1793.

Aminov, R.I., Garrigues-Jeanjean, N., Mackie, R.I., 2001. Molecular ecology of tetracycline resistance: development and validation of primers for detection of tetracycline resistance genes encoding ribosomal protection proteins. Appl. Environ. Microbiol. 67, 22–32.

Andersen, S.R., 1993. Effects of waste water treatment on the species composition and antibiotic resistance of coliform bacteria. Curr. Microbiol. 26, 97–103.

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

Baker-Austin, C., Wright, M.S., Stepanauskas, R., McArthur, J.V., 2006. Co-selection of antibiotic and metal resistance. Trends Microbiol. 14, 176–182.

Baquero, F., Martínez, J.-L., Cantón, R., 2008. Antibiotics and antibiotic resistance in water environments. Curr. Opin. Biotechnol. 19, 260–265.

Bellar, T.A., Lichtenberg, J.J., Kroner, R.C., 1974. Occurrence of organohalides in chlorinated drinking waters. J. Am. Water Works Ass. 66 (12), 703–706.

Billington, S.J., Songer, J.G., Jost, B.H., 2002. Widespread distribution of a *tet*(W) determinant among tetracycline-resistant isolates of the animal pathogen *Arcanobacterium pyogenes*. Antimicrob. Agents Chemother. 46, 1281–1287.

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

Diaz-Torres, M.L., Villedieu, A., Hunt, N., McNab, R., Spratt, D.A., Allan, E., Mullany, P., Wilson, M., 2006. Determining the antibiotic resistance potential of the indigenous oral microbiota of humans using a metagenomic approach. FEMS Microbiol. Lett. 258, 257–262.

Doherty, N., Trzcinski, K., Pickerill, P., Zawadzki, P., Dowson, C.G., 2000. Genetic diversity of the *tet*(M) gene in tetracycline-resistant clonal lineages of *Streptococcus pneumoniae*. Antimicrob. Agents Chemother. 44, 2979–2984.

Engemann, C.A., Keen, P.L., Knapp, C.W., Hall, K.J., Graham, D.W., 2008. Fate of tetracycline resistance genes in aquatic systems: migration from the water column to peripheral biofilms. Environ. Sci. Technol. 42, 5131–5136.

Grady, C.P.L.J., Daigger, G.T., Lim, H.C., 1999. Biological Wastewater Treatment. Marcel Dekker, New York.

Guardabassi, L., Petersen, A., Olsen, J.E., Dalsgaard, A., 1998. Antibiotic resistance in *Acinetobacter spp.* isolated from sewers receiving waste effluent from a hospital and a pharmaceutical plant. Appl. Environ. Microbiol. 64, 3499–3502.

Guardabassi, L., Wong, D.M.A.L.F., Dalsgaard, A., 2002. The effects of tertiary wastewater treatment on the prevalence of antimicrobial resistant bacteria. Water Res. 36, 1955–1964.

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

Levy, S.B., 2002. Active efflux, a common mechanism for biocide and antibiotic resistance. Soc. Appl. Microbiol. Symp. Ser. 31, 65S–71S.

Macovei, L., Zurek, L., 2006. Ecology of antibiotic resistance genes: characterization of *Enterococci* from houseflies collected in food settings. Appl. Environ. Microbiol. 72, 4028–4035.

Madigan, M.T., Martinko, J.M., 2006. Brock Biology of Microorganisms. Prentice Hall, Upper Saddle River, NJ.

Meckes, M.C., 1982. Effect of UV light disinfection on antibiotic-resistant coliforms in wastewater effluents. Appl. Environ. Microbiol. 43, 371–377.

Ng, L.K., Martin, I., Alfa, M., Mulvey, M., 2001. Multiplex PCR for the detection of tetracycline resistant genes. Mol. Cell. Probes 15, 209–215.

Nies, D.H., 2003. Efflux-mediated heavy metal resistance in prokaryotes. FEMS Microbiol. Rev. 27, 3–39.

Patterson, A.J., Rincon, M.T., Flint, H.J., Scott, K.P., 2007. Mosaic tetracycline resistance genes are widespread in human and animal fecal samples. Antimicrob. Agents Chemother. 51, 1115–1118.

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, 1685–1690.

Rice, L.B., 1998. Tn916 family conjugative transposons and dissemination of antimicrobial resistance determinants. Antimicrob. Agents Chemother. 42, 1871–1877.

Roberts, M., 1996. Tetracycline resistance determinants: mechanisms of action, regulation of expression, genetic mobility, and distribution. FEMS Microbiol. Rev. 19, 1–24.

Ruiz, N., Montero, T., Hernandez-Borrell, J., Vinas, M., 2003. The role of *Serratia marcescens* porins in antibiotic resistance. Microb. Drug Resist. 9, 257–264.

Salyers, A.A., Gupta, A., Wang, Y., 2004. Human intestinal bacteria as reservoirs for antibiotic resistance genes. Trends Microbiol. 12, 412–416.

Scott, K.P., Melville, C.M., Barbosa, T.M., Flint, H.J., 2000. Occurrence of the new tetracycline resistance gene *tet*(W) in bacteria from the human gut. Antimicrob. Agents Chemother. 44, 775–777.

Silver, S., Phung, L.T., 1996. Bacterial heavy metal resistance: new surprises. Annu. Rev. Microbiol. 50, 753–789.

Stepanauskas, R., Glenn, T.C., Jagoe, C.H., Tuckfield, R.C., Lindell, A.H., McArthur, J.V., 2005. Elevated microbial tolerance to metals and antibiotics in metal-contaminated industrial environments. Environ. Sci. Technol. 39, 3671–3678.

Storteboom, H.N., Kim, S.-C., Doesken, K.C., Carlson, K.H., Davis, J.G., Pruden, A., 2007. Response of antibiotics and resistance genes to high-intensity and low-intensity manure management. J. Environ. Qual. 36, 1695–1703.

Suzuki, M.T., Taylor, L.T., DeLong, E.F., 2000. Quantitative analysis of small-subunit rRNA genes in mixed microbial populations via 5′-nuclease assays. Appl. Environ. Microbiol. 66, 4605–4614.

Szczepanowski, R., Krahn, I., Linke, B., Goesmann, A., Puhler, A., Schluter, A., 2004. Antibiotic multiresistance plasmid pRSB101 isolated from a wastewater treatment plant is related to plasmids residing in phytopathogenic bacteria and carries eight different resistance determinants including a multidrug transport system. Microbiology 150, 3613–3630.