Contents lists available at ScienceDirect

Chemosphere

CrossMark

Influence of tetracycline on the microbial community composition and activity of nitrifying biofilms

Maria Matos [a,*], Maria A. Pereira [a], Pier Parpot [b], António G. Brito [a,d], Regina Nogueira [c]

[a] CEB – Centre of Biological Engineering, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal
[b] Centre of Chemistry, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal
[c] ISAH – Institute of Sanitary Engineering and Waste Management, University of Hannover, Welfengarten 1, D-30167 Hannover, Germany
[d] Institute of Agronomy, Department of Biosystems Sciences and Engineering, University of Lisbon, Tapada da ajuda, 1349-017 Lisboa, Portugal

HIGHLIGHTS

- Tetracycline did not affect the removal of carbon and nitrogen.
- The antibiotic affected the bacterial composition of the biofilms.
- The tetracycline removal was poor (28%).
- Biodegradation was probably the main removal mechanism of the antibiotic.
- The occurrence of tet(S) was influenced by the presence of tetracycline.

ARTICLE INFO

ABSTRACT

The present work aims to evaluate the bacterial composition and activity (carbon and nitrogen removal) of nitrifying biofilms exposed to 50 μg L^{-1} of tetracycline. The tetracycline removal efficiency and the occurrence of tetracycline resistance (tet) genes were also studied. Two sequencing batch biofilm reactors (SBBRs) fed with synthetic wastewater were operated without (SBBR1) and with (SBBR2) the antibiotic. Both SBBRs showed similar organic matter biodegradation and nitrification activity. Tetracycline removal was about 28% and biodegradation was probably the principal removal mechanism of the antibiotic. Polymerase chain reaction-denaturing gradient gel electrophoresis analysis of the bacterial community showed shifts leading to not only the fading of some ribotypes, but also the emergence of new ones in the biofilm with tetracycline. The study of the tet genes showed that tet(S) was only detected in the biofilm with tetracycline, suggesting a relationship between its occurrence and the presence of the antibiotic.

© 2014 Elsevier Ltd. All rights reserved.

1. Introduction

Antibiotics have been widely used therapeutically in human and veterinary medicine as well as non-therapeutically in animal production and many of them are excreted unchanged as active compounds into wastewater. The presence of antibiotics in wastewater and their persistence through wastewater treatment plants (WWTPs) potentially contribute to their release into surface waters, where they can accumulate and reach detectable and biologically active concentrations. Dissemination of antibiotics in the environment can lead to the emergence and spread of antibiotics resistance, which can reduce their therapeutic potential against pathogens, thus increasing risks to public health (Rizzo et al.,

2013). Due to all these facts, antibiotics are emerging as a new group of pollutants in wastewater and a significant number of studies focused on their presence and elimination in WWTPs have been reported in the literature as reviewed by Miège et al. (2009) and Le-Minh et al. (2010). According to them, the main mechanisms involved in the removal of antibiotics from wastewater are biodegradation, sorption onto extracellular polymeric substances (EPS), filtration and chemical oxidation. However, since WWTPs rely on the composition and activity of their microbial communities, it is also important to study if antibiotics in the wastewater might negatively affect the diversity and activity of the microbial communities in biological systems.

Biodegradation of organic matter by heterotrophic bacteria and biological oxidation of ammonia via nitrite to nitrate by autotrophic bacteria, a process known as nitrification, are key processes of the biological wastewater treatment. Typical heterotrophic

* Corresponding author. Tel.: +351 253 604414; fax: +351 253 678 986.
E-mail address: mariaines.matos@deb.uminho.pt (M. Matos).

bacteria in WWTPs are affiliated to the alpha, beta and gamma subclasses of the Proteobacteria (Seviour and Nielsen, 2010). Other members affiliated to the Bacteroidetes, Firmicutes and Actinobacteria phyla have also been frequently retrieved (Seviour and Nielsen, 2010). Autotrophic nitrifying bacteria in WWTPs encompass the ammonia oxidizing bacteria (AOB) that are almost exclusively restricted to the Betaproteobacteria and the nitrite oxidizing bacteria (NOB) that are distributed among the Alphaproteobacteria, in particular within the *Nitrobacter* genus and the phylum Nitrospirae, assigned to the *Nitrospira* genus (Daims and Wagner, 2010). There are a limited number of studies that report the effect of antibiotics in the community composition and activity of heterotrophs and nitrifiers in WWTPs. Collado et al. (2013) reported bacterial community shifts in a lab-scale sequencing batch reactor (SBR) fed with synthetic wastewater containing 50 μg L^{-1} of sulfamethoxazole, but they did not observe any impact in the removal of carbon and nitrogen as well as in the number of resistance genes. Deng et al. (2012) showed that the performance of a full-scale biosystem fed with wastewater containing high concentrations of antibiotics (mainly streptomycin at 3.96 mg L^{-1}) was maintained due to the adjustment of the bacterial, archaeal and eukaryal communities. Furthermore, they suggested that the increase of Betaproteobacteria and Bacteroides observed in the aerobic reactor of the biosystem was related with their ability to acquire antibiotic resistance genes. In contrast, Wunder et al. (2013) reported that ciprofloxacin (3.33 μg L^{-1}) can affect both the structure and activity of biofilms growing in a continuous-flow rotating annular bioreactor, while erythromycin and sulfamethoxazole (at similar concentration as ciprofloxacin) did not affect the environmental bacterial biofilms. Wunder and his co-workers also found that a mixture of the three antibiotics at 0.33 μg L^{-1} (for each compound) is unlikely to affect the performance of the biofilm process, at least in terms of degradation of easily biodegradable substrates.

The main goal of the present work was to evaluate the potential influence of tetracycline in the composition and activity of the microbial community (total and nitrifying bacteria) in biofilm reactors. The removal of tetracycline, the sorption of tetracycline onto EPS as well as the occurrence of tetracycline resistance (*tet*) genes were also studied. Tetracycline was the selected antibiotic because it is commonly detected in wastewater (Miège et al., 2009). For instance, Batt and Aga (2005) detected 0.62 μg L^{-1} of tetracycline in influent samples and Pailler et al. (2009) detected 1–85 μg L^{-1}. Furthermore, to the best of our knowledge, there are no studies on the effects of tetracycline at these relevant concentrations in WWTPs (from ng L^{-1} to a few μg L^{-1}) (Miège et al., 2009) in the composition and activity of wastewater treatment microbial communities. To achieve the main purpose of this study, two sequencing batch biofilm reactors (SBBRs) were operated for 60 d without (control unit) and with tetracycline at a concentration of 50 μg L^{-1}.

2. Materials and methods

2.1. Experimental setup

Two 1.5 L SBBRs were operated with a constant cycle time of 12 h. The SBBRs cycle consisted of a 5 min fill period, a 710 min aerated period, followed by a 5 min draw period. At the end of each cycle, 0.75 L of effluent was pumped out of the reactors. The biofilm was formed on polyethylene carriers (Matos et al., 2012) and the carrier concentration was 10% of the reactors working volume (V_T). The resulting volume exchange ratio was 0.56 and the hydraulic retention time (HRT) was 21.6 h. The reactors were inoculated with 5% of biofilm carriers collected from a laboratory SBBR enriched in nitrifying bacteria. 5% of new clean carriers were added

to complete the 10% of the reactors V_T. During the aerated phase, an airflow of 2 L min^{-1} was supplied through membrane diffusers. The applied airflow ensured that biofilm carriers were moving freely along with the liquid in the reactors. The composition of the synthetic wastewater simulated a municipal wastewater (Nopens et al., 2001). SBBR1 was operated without tetracycline (control), while in SBBR2 the synthetic wastewater was supplemented with the antibiotic (50 μg L^{-1}). The reactors were operated for 60 d. Regular cleaning of feed storage vessels, feed lines and reactors was performed to avoid the proliferation of microorganisms on tubes and walls.

2.2. Analytical methods

Total kjeldahl nitrogen (TKN), N–NH$_4^+$, N–NO$_2^-$, N–NO$_3^-$, total chemical oxygen demand (COD) and total suspended solids (TSS) were determined according to the Standard Method (APHA, 2005). Samples for N–NH$_4^+$, N–NO$_2^-$ and N–NO$_3^-$ analyses were immediately filtered through a 0.45 μm pore size filter (Advantec, Dublin, CA, USA). Biofilm concentration (mg carrier^{-1}) was estimated according to Matos et al. (2012). Tetracycline was determined by solid phase extraction (SPE) followed by high performance liquid chromatography–mass spectrometry (HPLC–MS). Before SPE extraction, samples were filtered through a 0.45 μm pore size filter. Then, the pH of the sample was adjusted to <3 with 40% H$_2$SO$_4$ and 50 μL of 5% Na$_2$EDTA as well as 50 μL of 50 μg mL^{-1} chlorotetracycline solution (internal standard) were added to the sample. The SPE procedure consisted of the precondition of an Oasis HLB cartridge (30 mg mL^{-1}, Waters, Miliford, MA, USA) with 1 mL of methanol, 1 mL of 0.5 N HCl and 1 mL of deionized water sequentially. Then, 5 mL of sample was extracted with the HLB cartridge at a flow rate of approximately 5 mL min^{-1} and the cartridge was washed with 1 mL of 5% methanol aqueous solution. Finally, the antibiotic was eluted with 1 mL of methanol. The extract was dried under a gentle stream of N$_2$ and the dried residue was dissolved with 1 mL of mobile phase B (0.1% acid formic in acetonitrile). The HPLC system consisted of a HPLC Pump (Finnigan Surveyor Plus), an autosampler (Finnigan) and a photodiode array detector (Finnigan). Tetracycline and the internal standard were separated using a Synergi Hydro-RP column (150 mm $\cdot$ 4.6 mm) packed with 4 μm size particles (Phenomenex, Torrance, CA, USA). A multistep binary elution gradient composed of 0.1% acid formic in water (mobile phase A) and in acetonitrile (mobile phase B) was pumped through the column with a flow rate of 0.3 mL min^{-1}. The separation of tetracycline and internal standard was achieved with the following mobile phase gradient program: at 0 min A/B = 50/50; 10 min A/B = 10/90 and 15 min A/B = 50/50. The MS system consisted of a linear ion trap mass spectrometer (Thermo Lxq) equipped with an electrospray ionization (ESI) source operated in the positive ionization mode. The optimal conditions for the ESI source, obtained with a standard solution of tetracycline, were 275 °C capillary temperature and 13 V capillary voltage. Selected ion monitoring mode was chosen to quantify the antibiotic.

2.3. Biofilm sampling

Biofilm samples were collected from both reactors at the end of the experiment. Two biofilm carriers were removed from the reactors and placed aseptically into a falcon tube containing 10 mL of sterile phosphate buffered saline solution (PBS). The tube was vigorously vortexed for 1 min. Subsequently, the clean carriers were removed and the biofilm suspension was immediately stored at −20 °C prior to DNA and EPS extraction or was fixed in 4% paraformaldehyde prior to fluorescence *in situ* hybridization (FISH).

2.4. EPS extraction

EPS were extracted according to Winkler et al. (2007). Briefly, 10 mL of biofilm suspension were centrifuged at 5000 rpm for 10 min and the supernatant was discarded. The biofilm pellet was then resuspended in 10 mL deionized water and placed into an oven at 80 °C for 10 min. The mixture was centrifuged while still hot at 7500 rpm for 20 min at room temperature. The supernatant was filtered through a 0.45 μm pore size filter and stored at −20 °C prior to tetracycline determination.

2.5. Tetracycline mass balance analysis

The tetracycline mass balance was done assuming that the elimination of tetracycline was achieved by sorption and biodegradation. Therefore, the metabolized tetracycline was calculated by the following equation:

$$M_d = M_i - M_e - M_s \qquad (1)$$

where M_d is the total mass of tetracycline degraded during the 60 d of experiment (μg), M_i is the total mass of tetracycline fed to the reactor during the 60 d of experiment (μg), M_e is the total mass of tetracycline discharged from the reactor during the 60 d of experiment and M_s is the mass of tetracycline sorbed on the EPS on day 60 (μg).

2.6. Microbial analysis

2.6.1. DNA extraction and amplification

DNA extraction was performed using a modified protocol of the PowerSoil DNA Isolation Kit (MO BIO, Carlsbad, CA, USA) (Matos et al., 2012). An aliquot of the biofilm suspension (10 mL) was centrifuged at 10000 rpm for 30 s at room temperature. The supernatant was discarded and approximately 250 mg of the pellet was used for DNA extraction.

Polymerase chain reactions (PCR) were conducted in a 50 μL reaction mixture according to Rodrigues et al. (2010). For analysis of the total bacterial community by denaturing gradient gel electrophoresis (DGGE), a single round PCR was performed using the primers 338f and 518r, targeting the V3 region of the bacterial 16S rRNA gene (Supplementary Material (SM), Table SM-1). A 40-base GC clamp (5′-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGG CACGGGGGG-3′) was attached to the primer 338f at the 5′ end. The bacterial 16S rRNA gene fragments were also amplified for cloning using the forward primer 27f and the universal primer 1492r (Table SM-1). To study the AOB and NOB community, the primer sets CTO189f-CTO654r, 27f-Nbac1050r and 27f-Nsipra705r (Table SM-1), targeting AOB β-Proteobacteria, *Nitrobacter* spp. and *Nitrospira* spp. 16S rRNA gene, respectively, were used for cloning and DGGE analyses. For DGGE, PCR products were re-amplified using primers 338f-GC and 518r. The PCR were run under the thermocycling conditions reported by the authors mentioned in Table SM-1.

The occurrence of *tet* genes was assessed using primers targeting seven different *tet* genes according to the PCR protocols of Ng et al. (2001) (Table SM-2). The *tet* genes study were *tet*(A), *tet*(C), *tet*(G) (efflux pump genes), *tet*(M), *tet*(S) [ribosomal protection proteins (RPP) genes] and *tet*(X) (enzymatic modification gene). These *tet* genes were selected because they have been reported to occur frequently in WWTPs (Zhang and Zhang, 2011). The PCR product of *tet*(S) was further cloned and sequenced (according to Section 2.6.3) to assess the diversity of this gene in the biofilm samples.

2.6.2. DGGE analyses

DGGE analyzes of the PCR products were done as previously described (Rodrigues et al., 2010). Denaturant gradient from 30% to 60% was used. DGGE gels were scanned in an Epson Perfection V750 PRO (Epson, USA) and the DGGE profiles were compared using the BIONUMERICS TM software package (version 5.0; AppliedMaths BVBA, Sint-Martens-Latem, Belgium). Similarity indices of the compared profiles were calculated from the densitometric curves of the scanned DGGE profiles using the Pearson product–moment correlation coefficient (Häne et al., 1993). The relative intensity of a band on a DGGE profile was estimated by measuring the intensity of the band relative to the intensity of all bands in the corresponding sample. The intensities of the bands on the gel tracks were given by peak height in the densitometric curve.

2.6.3. Cloning and sequencing of PCR-amplified products

PCR products were purified with the QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany) and cloned into *Escherichia coli* JM 109 (Invitrogen, Carlsbad, CA, USA) using the Promega pGEM-T Easy vector system I (Promega, Madison, WI, USA) according to the manufacturer's instructions. Ninety-six white-colored recombinant colonies were randomly selected per sample and screened by DGGE. For that purpose, a PCR was done on the cell lysates using the primer sets described above. The DGGE mobility of the amplicons was compared to the band pattern of the samples. The clones whose amplicons corresponded to bands in the biofilm community profile were selected for sequencing. The clones with sequences showing identical DGGE mobility were also selected for replicate sequencing. Plasmids of selected clones were purified using the QIAquick PCR purification kit and subjected to DNA sequence analysis. Sequencing reactions were performed by STAB vida (Caparica, Portugal) using SP6 (5′-ATTTAGGTGACACTATAG-3′) and T7 (5′-TAATACGACTCACTATAGG-3′) sequencing primers. The sequences were imported into the BioEdit v7.0.5.3 software package for assembly. Consensus sequences obtained were manually checked and corrected when necessary. Additionally, they were checked for potential chimera artefacts by the Mallard program v1.02. Similarity searches for the obtained sequences derived from the biofilm clones were performed using the NCBI Blast search program within the GenBank database (http://www.ncbi.nlm.nih.gov/blast/). The sequences were deposited in the GenBank database under accession numbers KF512516–KF512537.

2.6.4. FISH

In situ hybridization of cells in the biofilms was performed with fluorescently labelled rRNA-targeted oligonucleotide probes according to Nielsen (2009). The fluorescently labelled oligonucleotide probes used were NIT3 targeting *Nitrobacter* spp. and NTSPA712, specific for the genus *Nitrospira* (Table SM-3). The probes were synthesized by Thermo Fisher Scientific (Waltham, MA, USA) with Cy3 dye attached to the 5′ end. Total cells were detected by staining the samples with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma–Aldrich, St. Louis, MO, USA) at a final concentration of 0.2 mg mL^{-1}. The samples were observed at a fluorescence microscope Olympus BX51 (Olympus, Center Valley, PA, USA) and the images were acquired with a color camera Olympus DP71 using the B-Cell software (Olympus). The DAPI signal was recorded using an excitation wavelength of 365–370 nm and an emission long pass filter by 421 nm and the Cy3 signal was recorded using an excitation wavelength of 530–550 nm and an emission long pass filter by 591 nm. The EUBMIX probe [a mixture of EUB338-I, EUB338-II and EUB338-III (Table SM-3)], designed to target almost all bacteria, was used as a positive control. The absence of significant autofluorescence and unspecific binding were checked by FISH analyses without probes and with the NONEUB probe (Table SM-3) labelled with Cy3, respectively.

3. Results and discussion

3.1. Performance of the SBBRs

The performance of a SBBR processing synthetic wastewater containing tetracycline (SBBR2) was assessed and compared to the performance of a SBBR operated without the antibiotic (SBBR1). Table 1 presents the operating conditions and performance of the SBBRs at pseudo-steady-state, defined as a constant performance in terms of biofilm concentration and carbon and nitrogen concentration in the effluent.

Both reactors demonstrated high performance with effluent COD concentrations lower than 31 ± 6 mg L^{-1} in SBBR1 and 33 ± 3 mg L^{-1} in SBBR2. Similar effluent TSS concentrations (23 ± 6 mg L^{-1} and 20 ± 5 mg L^{-1} in SBBR1 and SBBR2, respectively) and biofilm concentrations (6.0 ± 0.1 mg carrier^{-1} and 6.2 ± 0.6 mg carrier^{-1} in SBBR1 and SBBR2, respectively) were obtained. Total nitrogen concentrations in the effluent of SBBR1 and SBBR2 were 49 ± 5 mg L^{-1} and 49 ± 3 mg L^{-1}, respectively, which were also similar. Analogously, no significant differences were observed in the effluent N–NH$_4^+$ and N–NO$_3^-$ concentrations since they were 17 ± 0 mg L^{-1} and 31 ± 4 mg L^{-1}, respectively in SBBR1 and 18 ± 1 mg L^{-1} and 30 ± 1 mg L^{-1}, respectively in SBBR2. The high level of N–NH$_4^+$ in the effluent of both systems suggests that only a portion of ammonium supplied was oxidized to nitrate. This result might be related to the formation of a heterotrophic layer on the top of the nitrifying biofilms that limited oxygen diffusion (Nogueira et al., 2002). The concentration of tetracycline in the effluent of SBBR2 reached 36 ± 2 μg L^{-1}, indicating poor tetracycline removal in the biofilm reactor (tetracycline removal of 28%). A literature review showed quite variable removal efficiencies of tetracycline in WWTPs probably reflecting different operational conditions (e.g., HRT, sludge retention time (SRT), food-to-microorganism ratio, reactor TSS concentration, pH and temperature) (Le-Minh et al., 2010; Rizzo et al., 2013). For instance, Spongberg and Witter (2008) reported a tetracycline removal of about 12% in a municipal WWTP and Karthikeyan and Meyer (2006) of 68–100% after the secondary wastewater treatment. Kim et al. (2005) obtained tetracycline removal higher than 78.4% in a lab-scale SBR spiked with 250 μg L^{-1} of tetracycline and found that sorption was the principal removal mechanism of the antibiotic. Kim et al. (2005) also found no evidence of tetracycline biodegradation during the biodegradability test. Zhou et al.

(2013) reported high tetracycline removal in a conventional activated sludge plant with chlorination (90.6%) and in an oxidation ditch plant with ultra-violet disinfection (72.4%). They also observed that the removal of tetracycline in the WWTP was mainly due to sorption onto sludge. In this study, 21.9 ± 0.4 μg g^{-1} biofilm of tetracycline were found in the EPS on day 60, which correspond to about 15.8 μg of tetracycline sorbed on the EPS of SBBR2 on day 60 (M_s). Furthermore, the tetracycline mass balance analysis indicated that about 1262-μg of tetracycline were removed by biodegradation (M_d). This value corresponded to about 99% of the tetracycline removed and suggests that biodegradation played the key role in the removal mechanism of tetracycline. The importance of biodegradation on the removal of this antibiotic was also shown by Shi et al. (2011) since they suggested that the removal of tetracycline in a nitrifying granular sludge was characterized by a quick sorption and a slow process of biodegradation. Both studies, our and the one conducted by Shi et al. (2011), operated biofilm systems (granular reactors are considered a special case of biofilm systems). These systems are characterized by having very long SRT and acclimation potential, which may have favoured the degradation process of tetracycline. The SRT in SBBR2 (Teichgräber et al., 2001) was about 21 d.

The results obtained showed that the effluent parameters (carbon and nitrogen) were similar in both reactors, suggesting that the concentration of tetracycline tested did not affect the activity of the heterotrophic and the nitrifying bacteria. Similarly, Prado et al. (2009) did not observe significant differences in the elimination of organic matter and ammonia (nitrification) before and after the injection of a high level of tetracycline (40 mg L^{-1}) in a semi-industrial membrane reactor. In addition, Shi et al. (2013) did not detect any effect of 10 mg L^{-1} of tetracycline in a conventional granular system, but in a nitrifying granular reactor, the presence of tetracycline led to nitrite accumulation. However, the concentration of tetracycline reported to affect the nitrification performance was much higher than the one reported in WWTPs (ng L^{-1} to μg L^{-1}) and used in this study.

3.2. Bacterial community analysis

In order to gain insights into the diversity and shifts induced by tetracycline in the bacterial community of biofilms, especially in the nitrifying population, a molecular approach combining PCR–DGGE, cloning and sequencing techniques was further used.

Table 1
Operating conditions and performance of the SBBRs at the steady state (average value ± standard deviation).

Reactor		SBBR1	SBBR2
Influent parameters	COD (mg L^{-1})	247 ± 170	
	TKN (mg N L^{-1})	53.5 ± 4.9	
	N–NH$_4^+$ (mg N L^{-1})	39.3 ± 9.9	
	N–NO$_2^-$ (mg N L^{-1})	0.75 ± 0.43	
	N–NO$_3^-$ (mg N L^{-1})	0.88 ± 0.47	
	TN[a] (mg N L^{-1})	55.1 ± 4.9	
	Tetracycline (μg L^{-1})	–	49.8 ± 5.8
Effluent parameters[b]	COD (mg L^{-1})	31 ± 6	33 ± 3
	TKN (mg N L^{-1})	17.5 ± 2.4	18.1 ± 3.4
	N–NH$_4^+$ (mg N L^{-1})	16.6 ± 0.4	17.6 ± 0.8
	N–NO$_2^-$ (mg N L^{-1})	0.02 ± 0.03	0.49 ± 0.37
	N–NO$_3^-$ (mg N L^{-1})	31.4 ± 3.5	30.1 ± 1.4
	TN[a] (mg N L^{-1})	48.9 ± 4.6	48.8 ± 2.7
	Tetracycline (μg L^{-1})	–	35.6 ± 2.2
	TSS (mg L^{-1})	23 ± 6	20 ± 5
Reactor parameters	Biofilm (mg carrier^{-1})	6.0 ± 0.1	6.2 ± 0.6
	EPS tetracycline (μg g^{-1} biofilm)	–	21.9 ± 0.4

[a] TN = TKN + N–NO$_2^-$ + N–NO$_3^-$.
[b] See Fig. SM-1.

Diversity and shifts in the bacterial community of biofilms were estimated from the DGGE patterns of the partial 16S rRNA genes amplified from two biofilm samples: biofilms SBBR1 and SBBR2, collected from the SBBR operated without and with tetracycline, respectively, at the end of the experiment (day 60). Fig. 1 depicts the obtained DGGE patterns.

The similarity indices between the DGGE patterns from SBBR1 and SBBR2 were 71.7%, 77.8% and 89.8% for the total bacterial community, AOB and *Nitrobacter* spp., respectively. These results suggest that the tetracycline concentration tested induced slightly shifts in these microbial communities. The DGGE patterns of *Nitrospira* spp. were not presented because no PCR products were obtained using the primer pair 27f-Nsipra705r. The results obtained by FISH were consistent with the DNA amplification data since FISH analyses in biofilm samples (SBBR1 and SBBR2) gave negative results with the probe NTSPA712, designed to target *Nitrospira*.

To get an insight into the identity of the bacterial community (total bacteria, AOB β-Proteobacteria and *Nitrobacter* spp.) represented in the DGGE patterns, 16S rRNA gene fragments of a representative biofilm sample (SBBR1) were amplified, cloned and sequenced. The sequencing results are summarized in Table 2 and their corresponding position in each DGGE pattern are shown in Fig. 1.

The sequence retrieved from the *Nitrobacter* community (clone Nbac.1) was closely related to the *Bradyrhizobium japonicum*, species belonging to Bradyrhizobiaceae family of the alpha subdivision of Proteobacteria. Bradyrhizobiaceae family includes, among other taxa, the genus *Nitrobacter*, which is known to be phylogenetically very closely related to the *B. japonicum* (Orso et al., 1994). In fact, the sequence retrieved from clone Nbac.1 also presented high similarity (99%) to the *Nitrobacter winogradskyi*. *In situ* hybridization of biofilm samples with probe NIT3 gave positive results (Fig. SM-2), confirming that *Nitrobacter* species were probably the most important bacteria for nitrite oxidation in the biofilm samples.

Clones AOB.1 to AOB.9 showed phylogenetic affiliation to typical AOB. Clone AOB.9 was closely related to *Nitrosospira* spp. and clones AOB.1 to AOB.8 were related to *Nitrosomonas* species. AOB of the genera *Nitrosomonas* and *Nitrosospira* are abundant in activated sludge, and are involved in the nitrogen cycle, oxidizing ammonium to nitrite (Daims and Wagner, 2010).

The comparison between the DGGE patterns of nitrifying bacteria from the biofilms without (SBBR1) and with tetracycline (SBBR2) showed that *Nitrobacter*-like bacteria represented by band Nbac.1 appeared as prominent band (i.e., more intense) in both biofilms (Fig. 1c). This result suggests that the predominance of this ribotype was not affected by tetracycline. Regarding AOB (Fig. 1b), several bands corresponding to sequences closely related to *Nitrosomonas*-like organisms (AOB.1, AOB.2 and AOB.8) and to *Nitrosospira* species (AOB.9) did not significantly change in relative intensity in the DGGE patterns from SBBR1 and SBBR2 (Table SM-4). The presence of other *Nitrosomonas*-like bacteria (AOB.3 and AOB.4), represented by prominent bands in the DGGE pattern from SBBR1 (with band relative intensity values of 16.4% and 22.6%, respectively), became weaker in the AOB community from SBBR2 (with 2.2% and 18.1%, respectively). These results suggest that tetracycline affected negatively the predominance of these *Nitrosomonas*-like bacteria in the AOB β-Proteobacteria community. On the contrary, the predominance of *Nitrosomonas*-like bacteria represented by band AOB.5 was favoured in the presence of tetracycline, as suggested by the increase in the relative intensity of this band in the DGGE pattern of the AOB community from SBBR2 (Table SM-4). Regarding *Nitrosomonas*-like bacteria represented by bands AOB.6 and AOB.7, they corresponded to very faint bands in all biofilm patterns. The DGGE/sequencing results of nitrifying bacteria suggest that the dominant AOB present in both biofilms (with and without tetracycline) were affiliated to the genera *Nitrosomonas* and *Nitrosospira* and the dominant NOB to the *Nitrobacter* genus. The nitrifying-like bacteria (Nbac.1 and AOB1 to AOB.9) were not detected in the bacterial community from SBBR1 and SBBR2 (Fig. 1), suggesting that they were not dominant bacteria in the SBBRs.

The clones retrieved from the total bacterial community of the SBBR1 were affiliated to the Proteobacteria and the Bacteroidetes groups. Within the beta subclass of Proteobacteria, clone B.8 was affiliated to the genus *Zooglea* and clone B.12 to the genus *Acidovorax*. *Acidovorax* and *Zooglea* spp. are usually present in large numbers in WWTPs. *Zooglea* spp. have been considered to play an important role in floc formation (Rosselló-Mora et al., 1995) and *Acidovorax* spp. have been reported as denitrifying bacteria (Heylen et al., 2008). Clone B.10 was also related to a denitrifying genus, specifically to *Xanthomonas*, which belongs to the gamma subdivision of Proteobacteria (Merzouki et al., 1999). Other clones were related to the alpha subdivision of Proteobacteria, specifically to *Sandarakinorhabdus* (clone B.3) and *Caulobacter* (clone B.6 and B.7). *Caulobacter* species have been isolated from activated sludge and play an important role in the carbon cycle, having the ability to metabolize organic materials available in low concentrations (MacRae and Smit, 1991). The genus *Sandarakinorhabdus* belongs to the Sphingomonadaceae family and it is considered that the widespread distribution of Sphingomonadaceae bacteria in the environment is due to its ability to utilize a wide range of organic compounds including recalcitrant contaminants (White et al., 1996). Clones B.1 and B.4 were related to the Bacteroidetes group, namely to *Flavobacterium* genera and to Sphingobacteriaceae family, respectively. Bacteroidetes are commonly assumed to be specialized in degrading high molecular weight compounds and to have a preference for growth attached to support materials (Fernández-Gómez et al., 2013), playing important roles in wastewater treatment (Wagner and Loy, 2002). Finally, clone B.5, B.9 and B.11 were closely related to *Acinetobacter*, *Rhodobacter* and *Dysgonomonas* genus, respectively but, they corresponded to very diffuse bands on the DGGE pattern.

The sequences retrieved from the DGGE pattern of SBBR1 total bacterial community revealed ribotypes closely related to those typical in wastewater treatment systems and included floc formers, denitrifying and bacteria degrading high molecular weight compounds. Furthermore, the sequences were affiliated to the alpha, beta and gamma subdivisions of the phyla Proteobacteria
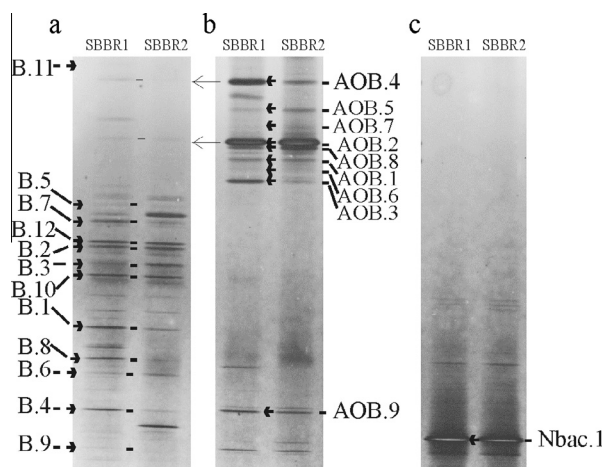

Fig. 1. DGGE patterns of total bacteria (a), AOB β-Proteobacteria (b) and *Nitrobacter* spp. (c) from SBBR1 and SBBR2 biofilm samples collected at the end of the experiment. Annotated DGGE bands were further identified by cloning and sequencing.

Table 2
Sequencing results of the selected clones.

Clone	Accession number	Sequence length (bp)	Closest relative (accession number, % sequence similarity)	Phylum
B.1	KF512516	1389	*Flavobacterium* sp. R-38296 (FR772064, 99%)	Bacteroidetes
B.2	KF512517	1492	Uncultured bacterium clone Q7563-HYSO (JN391976, 97%)	Bacteroidetes
B.3	KF512518	1446	Uncultured *Sphingomonadaceae* bacterium (AB478689, 98%) *Sandarakinorhabdus* sp. JC2488 (EU642848, 98%)	α-Proteobacteria
B.4	KF512519	1485	Uncultured bacterium clone KD6-86 (AY188323, 98%) Sphingobacteriaceae *bacterium* BR5-29 (EU370957, 96%)	Bacteroidetes
B.5	KF512520	1498	*Acinetobacter* sp. A12 (2011) (JN228276, 99%)	γ-Proteobacteria
B.6	KF512521	1444	Uncultured bacterium clone EMIRGE_OTU_s1t2b_6012 (JX222018, 97%) *Caulobacter* sp. BBCT22 (DQ337549, 97%)	α-Proteobacteria
B.7	KF512522	1443	Uncultured bacterium clone EMIRGE_OTU_s1t2b_6012 (JX222018, 97%) *Caulobacter* sp. BBCT22 (DQ337549, 97%)	α-Proteobacteria
B.8	KF512523	1497	Uncultured *Zoogloea* sp. clone W5S43 (GU560185, 99%) *Zoogloea* sp. A5 (DQ342276, 99%)	β-Proteobacteria
B.9	KF512524	1376	Uncultured bacterium clone SludgeB_bottom_83 (AB516144, 99%) *Rhodobacter* sp. KYW73 (FJ997595, 98%)	α-Proteobacteria
B.10	KF512525	1505	*Xanthomonas axonopodis* strain S53 (AB101447, 99%)	γ-Proteobacteria
B.11	KF512526	1425	Uncultured *Dysgonomonas* sp. clone NSBac30 (JX462548, 99%) *Dysgonomonas wimpennyi* (AY643492, 99%)	Bacteroidetes
B.12	KF512527	1487	Uncultured bacterium clone 7G8 (JF826429, 99%) *Acidovorax ebreus* TPSY strain (NR_074591, 98%)	β-Proteobacteria
AOB.1	KF512528	465	Uncultured *Nitrosomonas* sp. clone G67 (GQ891825, 97%) *Nitrosomonas* sp. DYS317 (AF363292, 97%)	β-Proteobacteria
AOB.2	KF512529	465	Uncultured bacterium clone 8AOB-R167 (KC346983, 96%) *Nitrosomonas* sp. DYS317 (AF363292, 96%)	β-Proteobacteria
AOB.3	KF512530	465	Uncultured bacterium clone 66-SIP10 (HQ116753, 99%) *Nitrosomonas eutropha* strain C91 (NR_074751, 98%)	β-Proteobacteria
AOB.4	KF512531	465	Uncultured bacterium clone 8AOB-R167 (KC346983, 97%)	β-Proteobacteria
AOB.5	KF512532	464	Uncultured bacterium clone 4R-11 (EU224353, 98%) *Nitrosomonas* sp. JL21 (AB000700, 96%)	β-Proteobacteria
AOB.6	KF512533	465	Uncultured bacterium clone 4R-11 (EU224353, 98%) *Nitrosomonas* sp. Is79A3 (CP002876, 97%)	β-Proteobacteria
AOB.7	KF512534	465	Bacterium enrichment culture clone 1.30 (GQ162356, 98%) *Nitrosomonas* sp. DYS317 (AF363292, 97%)	β-Proteobacteria
AOB.8	KF512535	465	Uncultured beta proteobacterium clone BF_4 (HM238151, 98%) *Nitrosomonas eutropha* strain C91 (NR_074751, 97%)	β-Proteobacteria
AOB.9	KF512536	465	Uncultured bacterium clone 19AOB-R390 (KC346994, 99%) *Nitrosospira* sp. DNB_E1 (AY138531, 99%)	β-Proteobacteria
Nbac.1	KF512537	986	Uncultured bacterium clone E33 (KC683250, 99%) *Bradyrhizobium japonicum* USDA 6 DNA (AP012206, 99%) *Nitrobacter winogradskyi* strain Nb-255 (NR_074324, 99%)	α-Proteobacteria

and Bacteroidetes. These groups of bacteria have been shown ability to acquire antibiotics resistance genes and they are generally related to the *tet* bacterial community. For instance, Li et al. (2013) reported that Proteobacteria, Actinobacteria and Bacteroidetes accounted for 88.4%, 9.21% and 2.33% of the total tetracycline resistant bacteria, respectively, in saline activated sludge and Li et al. (2010) found that the Gammaproteobacteria was the major class of tetracycline resistant bacteria isolated from a WWTP treating oxytetracycline manufacture effluents, followed by the Alphaproteobacteria and Betaproteobacteria. In this study the comparison of the DGGE patterns of total bacteria from biofilms without (SBBR1) and with tetracycline (SBBR2) showed that the bands corresponding to sequences related to *Caulobacter* (B.6), *Xanthomonas* (B.10) and *Acidovorax* (B.12) did not significantly change in intensity in the DGGE patterns from SBBR1 and SBBR2 (Table SM-4), suggesting that the relative abundance of these microorganisms in the total bacterial community was not affected by the antibiotic. In addition, the genus *Sandarakinorhabdus* (B.3) was found as dominant in the biofilms from SBBR2 (with band relative intensity value of 7.1%), but it was weaker in the biofilms from SBBR1 (with 1.4%), indicating that the relative abundance of these bacteria increased with the addition of tetracycline. Nevertheless, the DGGE/sequencing data also showed that *Flavobacterium* (B.1), Sphingobacteriaceae (B.4), other *Caulobacter*-related bacteria (B.7) and *Zooglea* (B.8) corresponded to strong bands in the DGGE pattern from SBBR1 (with band relative intensity values of 12.5%, 17.7%, 8.1% and 9.0%, respectively), while in the DGGE

pattern from SBBR2 they corresponded to very diffuse bands (with 4.6%, 2.9%, 3.3% and 0.7%, respectively). These results suggest that the predominance of these bacteria is strongly affected by the presence of tetracycline.

3.3. Occurrence of tet genes

The occurrence of 6 *tet* genes frequently detected in WWTPs was determined in the biofilms without (SBBR1) and with (SBBR2) tetracycline. The results are summarized in Table 3.

Among the 6 *tet* genes tested, the *tet*(M) gene encoding RPP was not detected in the biofilm from SBBR1 and SBBR2 sample, while the three efflux pump genes (*tet*(A), *tet*(C) and *tet*(G)) and the enzymatic modification gene (*tet*(X)) occurred in both biofilm. The results showed that the occurrence of *tet*(A), *tet*(C), *tet*(G) and *tet*(X) was independent of the presence of tetracycline. The RPP gene *tet*(S) was only detected in the presence of tetracycline (SBBR2) suggesting that the occurrence of this *tet* gene in the biofilms can be linked to the presence of the antibiotic. This finding

Table 3
PCR detection results of *tet* genes in the biofilms of the SBBRs (Fig. SM-2).

Reactor	Tetracycline resistant gene (*tet*)					
	A	C	G	M	S	X
SBBR1	+	+	+	−	−	+
SBBR2	+	+	+	−	+	+

suggests that *tet*(S) can potentially be an appropriate biomarket gene for tetracycline resistance capable of monitoring bacterial community. The emergence of the *tet*(S) in SBBR2 probably resulted from either or both, the enrichment of resistant bacteria and the transfer of the resistance gene between microbes (Rizzo et al., 2013). A Blast search of 30 *tet*(S) clones randomly selected showed that all sequences had 100% similarity to the known *tet*(S) carried out, among others, by *Listeria monocytogenes* (accession number: JX865374), *Streptococcus infantis* (JX275965) and *Enterococcus faecalis* (JN980097), belonging to the *Firmicutes* group. Since there are evidences linking the ever-increasing occurrence of antibiotic resistance genes in bacterial communities to the presence of antibiotics (Rizzo et al., 2013), further research should be done to quantify and study the expression of these *tet* genes in the biofilms without and with tetracycline.

The occurrence of *tet*(A), *tet*(C), *tet*(G), *tet*(X) and *tet*(S) in the bacterial community of the SBBR corroborate with the idea that the bacterial species of WWTPs effluents probably contain antibiotics resistance genes and antibiotics resistant bacteria. It is generally agreed that their release into the surface waters can reduce the therapeutic potential against pathogens, thus increasing risks to public health (Rizzo et al., 2013).

The results obtained in this study substantiate previous findings in the literature reporting that the concentration of antibiotics in wastewater (in ng L^{-1} to µg L^{-1} range) is not high enough to affect the performance of biological processes (Fan et al., 2009; Collado et al., 2013; Wang et al., 2014). This is probably explained by the ability of the microbial community to shift its composition towards the selection of those microbial populations that have resistance against antibiotics (Rizzo et al., 2013). Therefore, the shifts observed in this study in the microbial community might probably be attributed to a selection pressure produce by the presence of tetracycline. Probably, the bacteria impacted by tetracycline had no tetracycline resistance, while the bacteria that were not affected or were even enhanced by the antibiotic carried *tet* genes. Merlin et al. (2011) claims that biological treatment processes, particularly biofilm systems, are ideal environments for resistance emergence and spread because a high concentration of bacteria, sustained by a nutrient enrich environment, is continuously mixed with antibiotics at low concentrations. The results obtained corroborate with this idea since it was verified the emergence of *tet*(S) in the SBBR operated with 50 µg L^{-1} of tetracycline. However, based on our results it is not possible to ascertain the role of the biofilm in the appearance of resistance against tetracycline.

4. Conclusion

From this study it is suggested that tetracycline at a concentration of 50 µg L^{-1} did not significantly affect the removal of organic matter and nitrogen in the SBBRs, but influenced biofilm's bacterial composition. The fraction of *Flavobacterium*, *Caulobacter* and *Zooglea*-like bacteria and of a member of the Sphingobacteriaceae family decreased in the bacterial community, while the fraction of *Sandarakinorhabdus*-like bacteria increased. The analysis of the *tet* genes indicated a relationship between the occurrence of *tet*(S) and tetracycline contamination. On the contrary, the occurrence of *tet*(A), *tet*(C), *tet*(G) and *tet*(X) were observed in both biofilms without and with tetracycline.

Acknowledgements

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chemosphere.2014.06.094.

References

APHA, 2005. Standard Methods for the Examination of Water and Wastewater, 21st ed. American Public Health Association, Washington, DC.

Batt, A.L., Aga, D.S., 2005. Simultaneous analysis of multiple classes of antibiotics by ion trap LC/MS/MS for assessing surface water and groundwater contamination. Anal. Chem. 77, 2940–2947.

Collado, N., Buttiglieri, G., Marti, E., Ferrando-Climent, L., Rodriguez-Mozaz, S., Barceló, D., Comas, J., Rodriguez-Roda, I., 2013. Effects on activated sludge bacterial community exposed to sulfamethoxazole. Chemosphere 93, 99–106.

Daims, H., Wagner, M., 2010. The microbiology of nitrogen removal. In: Seviour, R., Nielsen, P.H. (Eds.), Microbial Ecology of Activated Sludge. IWA Publishing Company, London, pp. 95–126.

Deng, Y., Zhang, Y., Gao, Y., Li, D., Liu, R., Liu, M., Zhang, H., Hu, B., Yu, T., Yang, M., 2012. Microbial community compositional analysis for series reactors treating high level antibiotic wastewater. Environ. Sci. Technol. 46, 795–801.

Fan, C., Lee, P.K., Ng, W.J., Alvarez-Cohen, L., Brodie, E.L., Andersen, G.L., He, J., 2009. Influence of trace erythromycin and eryhthromycin-H$_2$O on carbon and nutrients removal and on resistance selection in sequencing batch reactors (SBRs). Appl. Microbiol. Biotechnol. 85, 185–195.

Fernández-Gómez, B., Richter, M., Schüler, M., Pinhassi, J., Acinas, S.G., González, J.M., Pedrós-Alió, C., 2013. Ecology of marine Bacteroidetes: a comparative genomics approach. ISME J. 7, 1026–1037.

Häne, B.G., Jäger, K., Drexler, H., 1993. The Pearson product–moment correlation coefficient is better suited for identification of DNA fingerprint profiles than band matching algorithms. Electrophoresis 14, 967–972.

Heylen, K., Lebbe, L., De Vos, P., 2008. *Acidovorax caeni* sp. nov., a denitrifying species with genetically diverse isolates from activated sludge. Int. J. Syst. Evol. Microbiol. 58, 73–77.

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

Kim, S., Eichhorn, P., Jensen, J.N., Weber, A.S., Aga, D.S., 2005. Removal of antibiotics in wastewater: effect of hydraulic and solid retention times on the fate of tetracycline in the activated sludge process. Environ. Sci. Technol. 39, 5816–5823.

Le-Minh, N., Khan, S.J., Drewes, J.E., Stuetz, R.M., 2010. Fate of antibiotics during municipal water recycling treatment processes. Water Res. 44, 4295–4323.

Li, D., Yu, T., Zhang, Y., Yang, M., Li, Z., Liu, M., Qi, R., 2010. Antibiotic resistance characteristics of environmental bacteria from an oxytetracycline production wastewater treatment plant and the receiving river. Appl. Environ. Microbiol. 76, 3444–3451.

Li, B., Zhang, X., Guo, F., Wu, W., Zhang, T., 2013. Characterization of tetracycline resistant bacterial community in saline activated sludge using batch stress incubation with high-throughput sequencing analysis. Water Res. 47, 4207–4216.

MacRae, J.D., Smit, J., 1991. Characterization of *Caulobacters* isolated from wastewater treatment systems. Appl. Environ. Microbiol. 57, 751–758.

Matos, M., Pereira, M.A., Nicolau, A., Rodrigues, A.L., Brito, A.G., Nogueira, R., 2012. Influence of carrier concentration on the control of *Galactomyces geotrichum* bulking and bacterial community of biofilm reactors. Desalination Water Treat. 41, 325–334.

Merlin, C., Bonot, S., Courtois, S., Block, J.C., 2011. Persistence and dissemination of the multiple-antibiotic-resistance plasmid pB10 in the microbial communities of wastewater sludge microcosms. Water Res. 45, 2897–2905.

Merzouki, M., Delgenès, J.P., Bernet, N., Moletta, R., Benlemlih, M., 1999. Polyphosphate-accumulating and denitrifying bacteria isolated from anaerobic–anoxic and anaerobic–aerobic sequencing batch reactors. Curr. Microbiol. 38, 9–17.

Miège, C., Choubert, J.M., Ribeiro, L., Eusèbe, M., Coquery, M., 2009. Fate of pharmaceuticals and personal care products in wastewater treatment plants – conception of a database and first results. Environ. Pollut. 157, 1721–1726.

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.

Nielsen, J.L., 2009. Protocol for fluorescence *in situ* hybridization (FISH) with rRNA-target oligonucleotides. In: Nielsen, P.H., Daims, H., Lemmer, H. (Eds.), FISH Handbook for Biological Wastewater Treatment: Identification and Quantification of Microorganisms in Activated Aludge and Biofilms by FISH. IWA Publishing, London, pp. 73–81.

Nogueira, R., Melo, L.F., Purkholdb, U., Wuertz, S., Wagner, M., 2002. Nitrifying and heterotrophic population dynamics in biofilm reactors: effects of hydraulic retention time and the presence of organic carbon. Water Res. 36, 469–481.

Nopens, I., Capalozza, C., Vanrolleghem, P.A., 2001. Stability analysis of a synthetic municipal wastewater. In: Technical Report, Department of Applied Mathematics, Biometrics and Process Control, University of Gent, Gent.

Orso, S., Gouy, M., Navarro, E., Normand, P., 1994. Molecular phylogenetic analysis of *Nitrobacter* spp. Int. J. Syst. Bacteriol. 44, 83–86.

Pailler, J.Y., Krein, A., Pfister, L., Hoffman, L., Guinard, C., 2009. Solid phase extraction coupled to liquid chromatography–tandem mass spectrometry analysis of

sulfonamides, tetracyclines, analgesics and hormones in surface water and wastewater in Luxembourg. Sci. Total Environ. 407, 4736–4743.

Prado, N., Ochoa, J., Amrane, A., 2009. Biodegradation by activated sludge and toxicity of tetracycline into a semi-industrial membrane bioreactor. Bioresour. Technol. 100, 3769–3774.

Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., Ploy, M.C., Michael, I., Fatta-Kassinos, D., 2013. Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: a review. Sci. Total Environ. 447, 345–360.

Rodrigues, A.L., Pereira, M.A., Janknecht, P., Brito, A.G., Nogueira, R., 2010. Biofilms formed on humic substances: response to flow conditions and carbon concentrations. Bioresour. Technol. 101, 6888–6894.

Rosselló-Mora, R.A., Wagner, M., Amann, R., Schleifer, K.H., 1995. The abundance of *Zoogloea ramigera* in sewage treatment plants. Appl. Environ. Microbiol. 61, 702–707.

Seviour, R.J., Nielsen, P.H., 2010. Microbial communities in activated sludge plants. In: Seviour, R., Nielsen, P.H. (Eds.), Microbial Ecology of Activated Sludge. IWA Publishing, London, pp. 95–126.

Shi, Y.J., Wang, X.H., Qi, Z., Diao, M.H., Gao, M.M., Xing, S.F., Wang, S.G., Zhao, X.C., 2011. Sorption and biodegradation of tetracycline by nitrifying granules and the toxicity of tetracycline on granules. J. Hazard. Mater. 191, 103–109.

Shi, Y., Xing, S., Wang, X., Wang, S., 2013. Changes of the reactor performance and the properties of granular sludge under tetracycline (TC) stress. Bioresour. Technol. 139, 170–175.

Spongberg, A.L., Witter, J.D., 2008. Pharmaceutical compounds in the wastewater process stream in Northwest Ohio. Sci. Total Environ. 397, 148–157.

Teichgräber, B., Screff, D., Ekkerlein, C., Wilderer, P.A., 2001. SBR technology in Germany – an overview. Water Sci. Technol. 43, 323–330.

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

Wang, S., Fan, C., Low, A., He, J., 2014. Comparison of microbial communities in sequencing batch reactors (SBRs) exposed to trace erythromycin and erythromycin-H_2O. Appl. Microbiol. Biotechnol. 98, 2667–2673.

White, D.C., Suttont, S.D., Ringelberg, D.B., 1996. The genus *Sphingomonas*: physiology and ecology. Curr. Opin. Biotechnol. 7, 301–306.

Winkler, G., Fischer, R., Krebs, P., Thompson, A., Cartmell, E., Griffin, P., 2007. Mass flow balances of triclosan in rural wastewater treatment plants and the impact of biomass parameters on the removal. Eng. Life Sci. 7, 42–51.

Wunder, D.B., Tan, D.T., LaPara, T.M., Hozalski, R.M., 2013. The effects of antibiotic cocktails at environmentally relevant concentrations on the community composition and acetate biodegradation kinetics of bacterial biofilms. Chemosphere 90, 2261–2266.

Zhang, X.X., Zhang, T., 2011. Occurrence, abundance, and diversity of tetracycline resistance genes in 15 sewage treatment plants across China and other global locations. Environ. Sci. Technol. 45, 2598–2604.

Zhou, L.J., Ying, G.G., Liu, S., Zhao, J.L., Yang, B., Chen, Z.F., Lai, H.J., 2013. Occurrence and fate of eleven classes of antibiotics in two typical wastewater treatment plants in South China. Sci. Total Environ. 452–453, 365–376.