



## Fate of antibiotic resistant cultivable heterotrophic bacteria and antibiotic resistance genes in wastewater treatment processes



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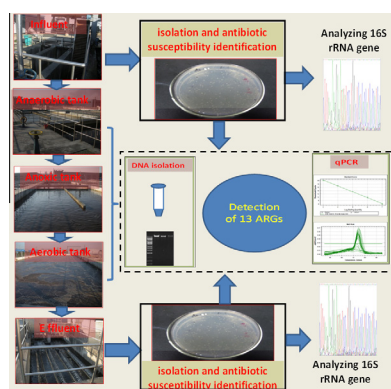
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### HIGHLIGHTS

- WWTPs contributed to removal of cultivable heterotrophic bacteria from sewage.
- Gram-negative and -positive bacteria dominated in the influent and effluent, respectively.
- The mean MAR index was lower in the influent than effluent samples.
- ARG abundance increased in the activated sludge after WWTP processes.

### GRAPHICAL ABSTRACT



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### ABSTRACT

Antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) are emerging contaminants of environmental concern. Heterotrophic bacteria in activated sludge have an important role in wastewater treatment plants (WWTPs). However, the fate of cultivable heterotrophic ARB and ARGs in WWTPs process remains unclear. In the present study, we investigated the antibiotic-resistant phenotypes of cultivable heterotrophic bacteria from influent and effluent water of three WWTPs and analysed thirteen ARGs in ARB and in activated sludge from anoxic, anaerobic and aerobic compartments. From each influent or effluent sample of the three plants, 200 isolates were randomly tested for susceptibility to 12 antibiotics. In these samples, between 5% and 64% isolates showed resistance to >9 antibiotics and the proportion of >9-drug-resistant bacteria was lower in isolates from effluent than from influent. Eighteen genera were identified in 188 isolates from influent ( $n=94$ ) and effluent ( $n=94$ ) of one WWTP. Six genera (*Aeromonas*, *Bacillus*, *Lysinibacillus*, *Microbacterium*, *Providencia*, and *Staphylococcus*) were detected in both influent and effluent samples. Gram-negative and -positive isolates dominated in influent and effluent, respectively. The 13 tetracycline-, sulphonamide-, streptomycin- and  $\beta$ -lactam-resistance genes were detected at a higher frequency in ARB from influent than from effluent, except for *sulA* and *CTX-M*, while in general, the abundances of ARGs in activated sludge from two of the three plants were higher in aerobic compartments than in anoxic ones, indicating abundant ARGs exit in the excess sledges and/or in uncultivable bacteria. These findings may be useful for elucidating the effect of WWTP on ARB and ARGs.

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## 1. Introduction

Numerous antibiotics have been extensively used to treat infectious diseases in humans and to promote the growth of food animals in agriculture (da Costa et al., 2008; Martinez, 2008). Residual antibiotics and their metabolites can be released from the body into the environment, causing chemical pollution (Gao et al., 2012). The practices of antibiotic use have influenced all the aspects of microbial genetic ecology, as revealed by the fact that antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) have been frequently detected in both liquids (wastewater, surface water, groundwater and even drinking water) (Schwartz et al., 2006; Storteboom et al., 2010) and in solids (sludge, soil and sediment) from the environment (Auerbach et al., 2007; Yang et al., 2014). Selective pressure from antibiotics can not only increase the concentrations of ARB by inhibiting antibiotic-susceptible bacteria, but also contributes to the selection of mutations and to horizontal gene transfer (HGT) between microorganisms (Martinez, 2008; Wang and Schaffner, 2011). Given that ARB and ARGs of clinical concern have been documented to arise from environmental sources (Martinez, 2008), greater attention has been focused on limiting resistance dissemination pathways between humans and the environment (Baquero et al., 2008).

Wastewater treatment plants (WWTPs) are supposed to hold an important place in the reduction or spreading of antibiotics and ARB (Iwane et al., 2001; Guardabassi et al., 2002; Munir et al., 2011). Although neither the links between the presence of residual antibiotics in WWTPs and the favouring of resistant bacteria nor the transfer of resistance at the low antibiotic concentrations in the environment have been well established (Martinez, 2008; Bouki et al., 2013), accumulated data show that WWTPs potentially provide an environment that has potential for the development and/or spread of resistance, as bacteria are continuously mixed with antibiotics at sub-inhibitory concentrations (Aminov et al., 2001; Auerbach et al., 2007; Davies, 2012). In addition, other factors in WWTPs, such as gene cassettes, integrons, plasmids, and heavy metals, play important roles in the exchange of resistance and contribute to resistance retention and dissemination in the WWTP systems (Stepanaukas et al., 2006; Zhang et al., 2011; Moura et al., 2012).

In WWTPs, activated sludge containing relatively stable, highly complex microbial communities has an important role in the removal of nutrients and pathogens from sewage. A recent report showed that there is a difference in the bacterial community structure and composition between aerobic and anaerobic sludge (Wang et al., 2013b). Activated sludge usually contains uncultivable bacteria that are difficult to study by traditional molecular methods, and 16S rRNA gene sequences from many bacteria cannot even be assigned to taxonomic groups at the phylum or class levels (Zhang et al., 2012). Currently, the majority of studies and reviews focusing on antibiotic resistance in WWTPs give special emphasis to faecal indicators and pathogens (including faecal coliforms (e.g. *Enterobacter*, *Klebsiella* and *Citrobacter*), enterococcal species, *Acinetobacter* spp. and *Staphylococcus* spp.) that are usually used to assess microbiological water quality and safety (Chen and Zhang, 2013; Łuczkiwicz et al., 2010; Korzeniewska et al., 2013; Rijal et al., 2009).

Current studies of antibiotic resistance in bacteria have typically focused on pathogens and faecal indicators such as *Enterobacteriaceae*, *Aeromonas* spp. (Igbinosa and Okoh, 2012), *Escherichia coli* (Reinthal et al., 2003) and *Acinetobacter* (Zhang et al., 2009), which only represent a small proportion of the total cultivable heterotrophic bacteria. Though pathogens do not produce antibiotics, they can harvest ARGs released from other

bacteria in environment. However, minimal is known about the characteristics and species of antibiotic-resistant heterotrophic bacteria, which dominate in activated sludge (Reinthal et al., 2003; Ferreira da Silva et al., 2007). Since most of the heterotrophic bacteria are uncultivable (Yang et al., 2014), qPCR methods are generally employed to investigate the ARGs at the samples level, including in samples from anaerobic and aerobic environment (Ma et al., 2011; Wang et al., 2013b) and WWTP sludge (Yang et al., 2014). However, the alterations in ARG concentrations under different bio-treatment processes remain unclear. Since the bacteria can obtain ARGs from the environment through horizontal gene transfer (HGT) and the sewage and sludge can be used on farm (Joao and Eddie, 2013), it is necessary to investigate effects of WWTPs on the ARGs and ARB.

The aims of the present study were to: (1) evaluate the alterations in antibiotic resistance of cultivable heterotrophic bacteria; (2) study whether the ARGs in the cultivable ARB from influent were different from those in effluent; and (3) investigate whether ARGs increased in compartments with the water flow. To accomplish these aims, the concentrations of cultivable heterotrophic bacteria were determined in the influent and effluent of three full-scale WWTPs (WWTPa, WWTPb, and WWTPc), and the susceptibility of these heterotrophic bacteria to 12 antibiotics was evaluated. The frequencies and concentrations of 13 ARGs were determined in the randomly selected isolates from influent and effluent, and in activated sludge samples from anaerobic, anoxic and aerobic compartments.

## 2. Materials and methods

### 2.1. Characteristics of wastewater treatment plants

Samples of influent, effluent and activated sludge were collected from WWTPa, WWTPb and WWTPc located in Wuxi, Jiangsu province, China. These plants have anaerobic, anoxic and aerobic treatment processes (Fig. S1). WWTPa has a secondary sedimentation tank, while a membrane bioreactor (MBR) is employed to replace the secondary sedimentation tank in WWTPb and WWTPc. WWTPa and WWTPb receive wastewater containing at least 75% domestic sewage and 25% pretreated industrial effluent, while WWTPc receives 70% domestic sewage and 30% pretreated industrial effluent. The parameters of these plants were provided in Table S1.

### 2.2. Sample collection

The influent samples (2 L) were collected after passing through the grille (Fig. S1). The effluent samples (2 L) were collected from the influent from sedimentation or MBR but not exposed to ultraviolet radiation or oxidizer (Fig. S1). Additionally, 3 L of activated sludge samples were collected from the anaerobic, anoxic and aerobic tanks of WWTPa and WWTPb. All these samples were collected in March (dry season) or July (wet season) 2012. Samples were kept on ice during transportation to the laboratory. Water samples of influent and effluent were immediately processed within 12 h. Sludge samples were centrifuged at 8000 rpm for 10 min at 4 °C, and the pellets were stored at –80 °C until DNA extraction.

### 2.3. Enumeration of cultivable heterotrophic bacteria and total coliforms

Total heterotrophs and total coliforms in the water samples were quantified by a dilution plating procedure (Korzeniewska et al., 2013). The samples were serially diluted using phosphate-

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