Science of the Total Environment 505 (2015) 704–711

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

tet genes as indicators of changes in the water environment: Relationships between culture-dependent and culture-independent approaches

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HIGHLIGHTS

- A molecular indicator of changes in water quality was identified.
- *E. coli* harboring the *tet*(*B*) gene were most abundant in wastewater and downstream river samples.
- Doxycycline concentrations were correlated with *tet*(*B*) levels.
- The concentration of *tet*(*B*) was correlated with other tetracycline resistance genes.

ARTICLE INFO

Article history:
Received 25 August 2014
Received in revised form 15 October 2014
Accepted 15 October 2014
Available online xxxx

Editor: D. Barcelo

Keywords:
Antibiotics
Tetracyclines
Bacteria
Resistance
Genes
Wastewater
River

ABSTRACT

The aim of this study was to identify tetracycline resistance determinants that could be used as molecular indicators of anthropogenic changes in aquatic environments. Two parallel approaches were used to examine the prevalence of *tet* genes: a culture-based method involving standard PCR and a method relying on quantitative PCR. The studied site was the Łyna River in Olsztyn (Poland). The culture-dependent method revealed that the concentrations of doxycycline-resistant bacteria harboring the *tet*(*B*) gene were higher in wastewater and downstream river samples than in upstream water samples. The *tet*(*B*) gene was transferred from environmental bacteria to *Escherichia coli*. The results generated by the culture-independent method validated statistically significant differences in *tet*(*B*) concentrations between upstream and downstream river sections, and revealed that *tet*(*B*) levels were correlated with the presence of other tetracycline resistance genes, dissolved oxygen concentrations, temperature and doxycycline concentrations in water. Our findings indicate that doxycycline-resistant bacteria, in particular *E. coli* harboring *tet*(*B*) or increased concentrations of *tet*(*B*), are potentially robust indicators of changes in water environments.

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1. Introduction

Thousands of tons of antibiotics have been administered to humans and animals since the discovery of penicillin. Antibiotics are never fully metabolized and are excreted as the parent compound or as its metabolites with urine and feces. Drug compounds from humans are excreted into wastewater (Kümmerer, 2009a,b). Those soluble compounds are not effectively removed during the treatment process in wastewater treatment plants (WWTPs) (Batt et al., 2006; Brown et al., 2006). They may reach surface waters where the concentrations of selected drugs can reach several μg/L (Jiang et al., 2011; Harnisz, 2013). When released into the environment, antibiotics and their transformation products can affect the composition and structure of aquatic microbial communities (Aminov and Mackie, 2007; Baran et al., 2011) that may be closely linked with ecosystem functions (Costanzo et al., 2005; Schmitt et al., 2004; Thiele-Bruhn and Beck, 2005). Antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) have also been identified as contaminants of emerging concern (Bouki et al., 2013; LaPara et al., 2011; Munir et al., 2011; Pruden et al., 2006), and they may enter ecosystems with treated wastewater. The presence of bacteria resistant to nearly all known antibiotics has been confirmed

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http://dx.doi.org/10.1016/j.scitotenv.2014.10.048
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in strongly anthropogenized environments (Kümmerer, 2004). The abovementioned creates a potential health risk for humans and animals because ARGs and ARB evacuated into the environment can be transferred back to humans and animals.

Antibiotic resistance is determined by genes located on the bacterial chromosome or mobile elements, such as plasmids, transposons and integrons (Marti et al., 2013; Mokracka et al., 2012). Therefore, the transfer of antibiotic resistance can be attributed mainly to conjugation, whereas transformation and transduction of resistance are usually more limited. Conjugation of broad-host-range plasmids supports the transfer of DNA across genus and species boundaries (Aminov, 2011). Information about horizontal gene transfer in aquatic ecosystems is generally provided by laboratory studies. Although laboratory studies have limited ability to reproduce natural aquatic systems and omit the role of environmental factors that can potentially affect gene transfer, they are a valuable source of information about possible trends in natural environments.

Wastewater treatment plants (Gao et al., 2012; LaPara et al., 2011; Munir et al., 2011), and animal farms (Ji et al., 2012; Koike et al., 2007; Peak et al., 2007; You et al., 2012) are possible sources of ARGs and ARB in the environment. The occurrence and fate of ARGs can be tested by two methods: a standard method that involves cultivation of resistant bacteria and identification of ARGs in isolates, and a culture-independent method where ARGs are directly quantified from environmental samples. In the latter approach, resistance genes are quantified with the use of a modern tool, namely real-time quantitative polymerase chain reaction (qPCR). This approach could give the impression that reliable results can be obtained only with the use of molecular methods and that the results generated by culture-dependent and culture-independent methods are not correlated. The culture-independent approach also has several drawbacks. It does not support the identification of drug-resistant bacteria or determinations of their multiresistance and pathogenicity. This method is also not suitable for less well equipped commercial laboratories.

There is a lack of information on the relationships between cultivable and non-cultivable antibiotic-resistant bacteria. This paper tests the hypothesis that although cultivable drug-resistant bacteria represent only a small fraction of total resistant bacteria in the environment, culture-dependent and independent methods can generate complementary results regarding drug resistance. For this reason, both approaches were applied in the study.

Our previous studies (Harnisz, 2013) demonstrated that wastewater evacuated from a municipal treatment plant affected the composition of native bacterial communities in the river and that doxycycline-resistant bacteria were potentially robust indicators of anthropogenic stress in river waters. The aim of this study was to identify tetracycline resistance determinants that could be used as molecular indicators of changes in aquatic environments. To demonstrate the complementarity of culture-dependent and culture-independent methods, both approaches were used to examine the prevalence of *tet* genes and their potential use as indicators of water quality.

2. Materials and Methods

2.1. Study Sites and Sampling

The plant's process line comprises mechanical, biological and chemical treatment sections and sludge processing units. The plant has the following technical parameters: treatment system — activated sludge, average processing capacity — 60,000 m^3/d, wastewater type — municipal wastewater, mechanical treatment devices — screenings, grit chamber and pre-sedimentation tank, biological treatment devices — separation chambers, aeration chambers and secondary sedimentation tanks, and sedimentation devices — closed and open digestion chambers, belt filter press and incinerator. Treated effluent is evacuated to the Łyna River.

The Łyna River is one of the largest watercourses in north-eastern Poland which is referred to as the Green Lungs of Poland. Glińska-Lewczuk (2006) divided the river into two sections based on changes in water quality parameters: the upper, unpolluted section extending from headwaters to Olsztyn, and the lower section with anthropogenic, urban influences.

Samples of river water from river sections upstream and downstream of the wastewater discharge point and samples of treated wastewater were collected in October and December 2010 and in January, March, May, July, September and October 2011. Upstream river water (URW) and downstream river water (DRW) were sampled approximately 600 m away from the treated wastewater discharge point (TWW) (N = 53°49′7.27″ and E = 20°26′57.95″). A total of 8 URW, 8 TWW and 8 DRW samples were collected during the study. Water and wastewater samples were collected into sterile bottles, transported to the laboratory at the temperature of 4 °C and processed on the day of collection.

Tetracycline concentrations in the studied samples were described in a previous study (Harnisz, 2013). Tetracycline concentrations were used to evaluate the correlations between *tet* genes and chemical pollution of water.

2.2. Experimental Design

Based on our previous experiments (Harnisz et al., 2011; Harnisz, 2013), tetracycline-resistant bacteria were chosen as indicators of changes in natural and anthropogenically altered waters. In this study, two parallel approaches were used to examine the prevalence of *tet* genes: a culture-based method and a culture-independent method.

The experimental analyses comprised: (I) tetracycline-(oxytetracycline- and doxycycline-)resistant isolates obtained from samples of river water and treated effluent; (II) environmental DNA extracted from the same samples. Resistant isolates were identified, and their *tet* resistance genes ((*A*), (*B*), (*C*), (*D*), (*E*), (*G*), (*K*), (*L*), (*M*), (*O*), (*S*), (*Q*) and (*X*)) were determined by standard polymerase chain reaction (PCR). The concentrations of *tet* genes ((*A*), (*B*), (*L*), (*M*), (*O*), (*Q*), (*X*)) and 16S rRNA genes were determined by quantitative PCR (qPCR) in environmental DNA (Fig. S1).

2.3. Culture-based Approach

2.3.1. Cultivation of Tetracycline-resistant Bacteria

Populations of resistant bacteria were cultivated on plates containing the TSA medium (Oxoid) with the addition (16 mg/L) of oxytetracycline or d\oxycycline (Sigma), incubated at 30 °C for 24 h. Antimicrobial doses were determined according to Clinical and Laboratory Standard Institute guidelines (CLSI, 2010). A total of 252 predominantly oxytetracycline-resistant strains (OTC^R) and 163 predominantly doxycycline-resistant strains (DOX^R) were isolated, purified and stored in TSB with 15% glycerol at the temperature of −80 °C for further tests.

2.3.2. Genomic DNA Extraction

A loopful of bacterial colonies harvested from agar plates was suspended in 0.5 mL of sterile water and heated at 95 °C for 10 min. After centrifugation at 5000 rpm for 5 min at 4 °C, the concentration and quality of extracted DNA were determined by microspectrophotometry (NanoDrop® ND-1000, NanoDrop Technologies, Wilmington, DE). If DNA quality was low, DNA was extracted again using the CTAB method (Korzeniewska and Harnisz, 2013). Genomic DNA was extracted in triplicate and stored at −20 °C for further analysis.

2.3.3. Identification of Isolates and ARGs

OTC^R and DOX^R isolates were identified by 16S rRNA gene sequencing. Universal primers 27F and 1492R were used to amplify nearly full-length 16S rRNA gene sequences according to a previously described

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