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Total resistance of native bacteria as an indicator of changes in the water environment

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ABSTRACT

This study analyzes changes in the total (intrinsic and acquired) resistance of autochthonous bacteria in a river which is a receiver of treated wastewater. In the analyzed samples, tetracycline contamination levels were low and characteristic of surface water bodies. An increase in the populations of tetracycline-resistant and fluoroquinolone-resistant microorganisms was noted in downstream river water samples in comparison with upstream river water samples, but the above trend was not observed in bacteria resistant to macrolides and β-lactams. The counts of doxycycline-resistant bacteria (DOX^R) were significantly correlated with doxycycline levels. The minimum inhibitory concentrations (MICs) for doxycycline in DOX^R isolates were higher in downstream river water than in upstream river water samples. The discharge of treated wastewater had no effect on the multi-drug resistance of oxytetracycline-resistant and doxycycline-resistant isolates. The results of the experiment indicate that the presence of doxycycline-resistant bacteria is a robust indicator of anthropogenic stress in river water.

1. Introduction

It was nearly 100 years ago that Alexander Fleming discovered penicillin. Since this breakthrough moment in medicine, thousands of tons of antibiotics have been administered to humans and animals. Both unchanged and metabolized antibiotics are discharged to wastewater with feces, and they can find their way to surface waters that receive treated effluents (Hoa et al., 2011; Kümmerer, 2009). In most aquatic ecosystems, antibiotic concentrations are low (Hoa et al., 2011; Jiang et al., 2011; Zou et al., 2011), but in the long-term perspective, sub-threshold doses of those drugs can increase bacterial resistance to antibiotics (Baquero et al., 2008; Hoa et al., 2011; Quinlan et al., 2011).

Antibiotic resistance can be both intrinsic and acquired. Intrinsic resistance is encoded in the bacterial genome, whereas acquired resistance surfaces in response to changes in bacterial DNA. *Aeromonas hydrophila* is intrinsically resistant to ampicillin, and this trait is used to culture the discussed microorganism on selective media. Examples of acquired resistance include methicillin resistance in *Staphylococcus aureus* (MRSA) and the production of antibiotic-resistant *Klebsiella pneumoniae* carbapenemase (KPC). Resistance is passed through the transfer of genes located on plasmids, transposons and integrons which are efficient vectors for the spread of antibiotic resistance genes between autochthonous bacteria (Davison, 1999). Wastewater treatment plants, in particular activated sludge chambers and biological filters, are hot spots for gene transfer (da Costa et al., 2006; Szczepanowski et al., 2009; Zhang et al., 2009) due to the availability of nutrients, supportive temperature, high density of microbial communities, presence of donors and recipients, as well as factors that contribute to selective pressure (Seveno et al., 2002).

Treated wastewater discharged to a body of surface water modifies its environmental parameters, including temperature, pH, dissolved oxygen and xenobiotic concentrations. For this reason, native bacterial communities in surface waters which are receivers of treated wastewater may undergo both quantitative and qualitative change. The presence of compounds which inhibit microbial growth, such as heavy metals and antibiotics, may suppress metabolic pathways (Costanzo et al., 2005). LaPara et al. (2011) demonstrated that wastewater treatment plants are sources of human-related *Bacteroides* and resistance genes in the natural environment, and that they play an important role in the global ecology of antibiotic resistance. According to Lupo et al. (2012), drug-resistance is a natural mechanism in bacteria, but river pollution could significantly contribute to microbial drug resistance.

Goñi-Urriza et al. (2000) have argued that in environmental studies, intrinsic antimicrobial resistance should be differentiated from acquired resistance through the use of selected drugs which

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determine the antibiotic resistance of pathogens and potential pathogens in aquatic environments, such as the use of β-lactams against *E. coli*. The above approach has been adopted by many researchers (da Costa et al., 2006; Reinthaler et al., 2003; Tao et al., 2010; Zhang et al., 2009). This study evaluates the total antibiotic resistance of native bacteria present in an aquatic environment. The analyzed microorganisms manifest both intrinsic and acquired resistance, and the aim of this experiment was to observe changes in the drug resistance profiles of autochthonous bacteria under the influence of treated effluents discharged to a river. The drug resistance of native bacteria has been investigated by Hoa et al. (2011), Huang et al. (2012) and Li et al. (2010), yet according to the author's best knowledge, this paper makes a pioneering contribution to Central and Eastern European research into antimicrobial resistance.

The main objective of this study was to investigate changes in the total resistance (intrinsic and acquired) (Moore et al., 2010) of autochthonous bacteria in a river which receives treated wastewater. The experiment comprised several research tasks: 1) to determine the size of microbial populations resistant to eight drugs of four antibiotic classes in view of the physical and chemical parameters of the studied water body, 2) to determine antibiotic concentrations in water and treated effluent samples, 3) to identify the level of resistance and multiple drug resistance of the analyzed isolates.

The total resistance of bacterial communities was tested with the use of drugs which are commonly used in human and veterinary medicine in Poland, including macrolides, β-lactams, tetracyclines and fluoroquinolones. The results of preliminary analyses (data not published) indicate that the effluents discharged into the studied river carry large quantities of tetracycline-resistant bacteria. Three tetracyclines were selected for determinations of the antibiotic content of water samples. A statistical analysis of the results of this work confirmed that the size of bacterial populations resistant to tetracyclines could be indicative of changes in water bodies that receive treated wastewater, therefore, tetracycline-resistant isolates were selected for further analysis of resistance and multi-drug resistance.

2. Materials and methods

2.1. Study sites and sampling

Effluent samples were collected from a secondary sedimentation tank of the Łyna Wastewater Treatment Plant (WWTP) and from the Łyna River in Olsztyn, Poland. The examined WWTP with the capacity of 60 000 m^3/d comprises mechanical and biological treatment facilities. The plant treats mostly household wastewater. The treatment process involves preliminary treatment (screening and grit removal), primary treatment (gravity sedimentation tanks) and secondary treatment (activated sludge) followed by secondary sedimentation. Treated effluent is discharged into the Łyna River.

Samples of river water from upstream and downstream sections 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. Samples of upstream river water (URW) and downstream river water (DRW) were collected approximately 600 m from the discharge point of treated wastewater (TWW) (N 53°49′7.27″, E 20°26′57.95″). The samples were placed in sterile bottles, transported to the laboratory at a temperature of 4 °C and processed on the day of collection.

2.2. Physicochemical parameters

Physicochemical parameters of river water and treated wastewater samples, including temperature (°C), oxygen concentrations (mg/L) and pH, were determined with the use of the Hydrolab Multiprobe 12 (Scott).

2.3. Antibiotic analyses

The concentrations of tetracyclines (tetracycline, oxytetracycline and doxytetracycline) were determined by tandem mass spectrometry with high-performance liquid chromatography (HPLC–MS/MS) which was performed after solid-phase extraction (SPE).

URW, DRW and TWW samples were left on the laboratory table to achieve room temperature. 300 mL of each sample was acidified to pH 2.5 with the use of orthophosphoric acid. The samples were enriched by SPE using Oasis HLB cartridges (10 mg/1 mL, Waters). SPE cartridges were preconditioned with 3 × 2 mL of acetonitrile (ACN), 3 × 2 mL of ACN/water (1/1 v/v), 2 × 2 mL of ultrapure water and 3 × 2 mL of ultrapure water with a pH 2.5. 300 mL of each water sample was passed through the cartridges (flow rate 1 mL/min). The analytes were eluted with 3 mL mixture of ACN/water (1:1) containing 0.1% trifluoroacetic (TFA) acid, evaporated to dryness and redissolved in 1 mL of elution solvent.

HPLC analyses were performed using the Agilent 1200 system. Antibiotics were separated on a reverse-phase column (Xterra MS C18) which was operated at 15 °C with the flow rate of 0.5 mL/min. Injection volume was 5 μL. Mobile-phase solvents were eluent A – ultrapure water (95%) ACN (5%), TFAA (0.1%), and eluent B – ACN, TFAA (0.1%), by using of a gradient elution program. The gradient profile started at 100% A for 1 min, it changed to 50% A for 6 min, returned to 100% A for 0.5 min and was kept constant at 100% A for 5.5 min. The presence of antibiotics was detected using a hybrid quadrupole/linear ion trap tandem mass spectrometer (Applied Biosystems 4000 Q TRAP) with electrospray ionization. The analyses were performed in the positive-ion mode.

Calibration solutions were prepared by diluting intermediate solutions in ACN/H2O (1/1 v/v) in the concentration range of 0.005–1 μg/mL. Based on the analyzed standard samples, calibration curves were developed to present the correlations between the analyte peak area and analyte concentrations in the extract obtained after SPE enrichment. Calibration curves coefficients in the studied concentration range were characterized by a high degree of linear correlation (R^2 = 0.9992–0.9998).

2.4. Heterotrophic plate counts and counts of antibiotic resistant bacteria

To obtain 30–300 colony forming units (CFU) per plate, TWW samples were diluted with saline water, and URW and DRW samples were passed through a cellulose filter (pore diameter 0.45 μM, Millipore) or diluted with saline water. Greater accuracy was achieved by plating triplicates. Heterotrophic plate counts (HPC) were determined on plates containing the TSA medium (Oxoid). The plates were cultured at 30 °C for 24 h. The size of resistant populations of native bacteria was determined on plates containing the TSA medium (Oxoid) with the addition of antibiotics (Sigma). The following antibiotics were selected for the test: tetracyclines (oxytetracycline, doxytetracycline) β-lactams (amoxicillin, cefuroxime), fluoroquinolones (norfloxacin, enrofloxacin) and macrolides (roxithromycin, erythromycin). Those antimicrobials are widely used in Poland (http://www.esac.ua.ac.be/main.aspx?c=*ESAC2&n=50279). Antimicrobial doses were determined in accordance with Clinical and Laboratory Standard Institute guidelines (CLSI, 2010). The applied doses were: 1 mg/L for erythromycin, 4 mg/L for roxithromycin, 8 mg/L for amoxicillin, 16 mg/L for tetracyclines and fluoroquinolones and 32 mg/L for cefuroxime. Resistant microorganisms were incubated at 30 °C for 24 h.

Cultured colonies of HPC and bacteria resistant to oxytetracycline (OTC^R), doxytetracycline (DOX^R), amoxicillin (AMO^R), cefuroxime (CEF^R), norfloxacin (NOR^R), enrofloxacin (ENR^R), roxithromycin (ROX^R) and erythromycin (ERY^R) were counted, and the results were stated in terms of CFU per ml of river water or treated wastewater. A total of 252 of OTC^R and 163 of DOX^R dominated colonies were selected for further tests. They were purified on the TSA medium with oxytetracycline or doxytetracycline (16 mg/L) and stored in TSB (Oxoid Ltd.) with glycerol at a temperature of −80 °C.

2.5. Minimum inhibitory concentrations

Minimum inhibitory concentrations (MICs) of oxytetracycline and doxycycline were determined by the agar dilution method according to CLSI guidelines (2010), with final antibiotic concentrations in the range of 16–512 μg/mL.

2.6. Antibiotic susceptibility testing

OTC^R and DOX^R isolates were subjected to sensitivity testing against 12 antimicrobials: tobramycin (TOB 10 μg), amoxicillin/clavulanic acid (AMC 75 μg), mezlocillin (MEZ 75 μg), piperacillin (PRL 75 μg), ceftazidime (CAZ 30 μg), cefotaxime (CTX 30 μg), tetracycline (TE 30 μg), tigecycline (TGC 15 μg), ciprofloxacin (CIP 5 μg), enrofloxacin (ENR 5 μg), norfloxacin (NOR 10 μg) and trimethoprim/sulfamethoxazole (SXT 1.25/23.75 μg). Standard methods were applied (Bauer et al., 1966). All disks were supplied by Oxoid. Resistance was estimated by measuring the growth inhibition zone according to CLSI guidelines (2010).

2.7. Data analyses

Statistical analyses were carried out using the STATISTICA 10 software package (StatSoft Inc., 1984–2011). One-way analysis of variance (ANOVA) was performed to determine variations in the abundance of the studied bacterial groups and antibiotic concentrations in samples from different sites. The correlations between