Chemosphere 128 (2015) 134–141

Contents lists available at ScienceDirect

Chemosphere

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

The impact of a freshwater fish farm on the community of tetracycline-resistant bacteria and the structure of tetracycline resistance genes in river water

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HIGHLIGHTS

- The impact of fish farm on the structure of ARB and ARGs in river water was assess.
- Culture-dependent survey revealed no significant differences in the abundance of ARB.
- No significant differences in the frequency of *tet* genes were observed.
- The fish farm's impact on river water was manifested only by increased diversity of ARGs.

ARTICLE INFO

Article history:
Received 29 September 2014
Received in revised form 30 December 2014
Accepted 12 January 2015
Available online 17 February 2015

Handling Editor: A. Gies

Keywords:
Freshwater fish farm
Tetracyclines
Antibiotic resistant bacteria (ARB)
Antibiotic resistant genes (ARGs)

ABSTRACT

The aim of this study was to assess the impact of a fish farm on the structure of antibiotic resistant bacteria and antibiotic resistance genes in water of Drwęca River. Samples of upstream river waters; post-production waters and treated post-production waters from fish farm; as well as downstream river waters were monitored for tetracycline resistant bacteria, tetracycline resistant genes, basic physico-chemical parameters and tetracyclines concentration. The river waters was characterized by low levels of pollution, which was determined based on water temperature, pH and concentrations of dissolved oxygen and tetracycline antibiotics. Culture-dependent (heterotrophic plate counts, counts of bacteria resistant to oxytetracycline (OTC^R) and doxycycline (DOX^R), minimum inhibitory concentrations for oxytetracycline and doxycycline, multidrug resistance of OTC^R and DOX^R, qualitative composition of OTC^R and DOX^R, prevalence of *tet* genes in resistant isolates) and culture-independent surveys (quantity of *tet* gene copies) revealed no significant differences in the abundance of antibiotic-resistant bacteria and antibiotic resistance genes between the studied samples. The only way in which the fish farm influenced water quality in the Drwęca River was by increasing the diversity of tetracycline-resistance genes. However, it should also be noted that the bacteria of the genera *Aeromonas* sp. and *Acinetobacter* sp. were able to transfer 6 out of 13 tested *tet* genes into *Escherichia coli*, which can promote the spread of antibiotic resistance in the environment.

1. Introduction

In recent decades, a significant increase in global aquaculture production was accompanied by intensified use of antimicrobials to treat bacterial infections (Boxall, 2010). Cabello et al. (2013) reported that 80% antibiotics used in aquaculture enter the water environment. Antibiotic concentrations in the ecosystem are high enough to exert selective pressure on bacteria by completely or partially inhibiting wild-type (sensitive to antimicrobials) bacterial populations (Tello et al., 2012). This process alters biodiversity in aquatic environments and the microbiota characteristic of water animals.

Antibiotic-resistant bacteria are increasingly often found in the vicinity of fish farms where antimicrobials are used, which points to a causal relationship between these two phenomena (Guardabassi et al., 2000; Schmidt et al., 2000). Bacterial resistance to antibiotics is determined by genes located on the chromosome or mobile genetic elements, such as plasmids, transposons and integrons (Korzeniewska and Harnisz, 2013), which facilitates their transfer between different bacterial species. In view of the above, antibiotic-resistant bacteria (ARB) and antibiotic resistance genes

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http://dx.doi.org/10.1016/j.chemosphere.2015.01.035
0045-6535/

(ARGs) have been identified as contaminants of emerging concern (Pruden et al., 2006). A knowledge of the sources and mechanisms of ARB and ARG dissemination is required because their proliferation and transfer poses a serious public health risk. The knowledge will support the development of effective strategies for controlling antibiotic resistance and assessing human health risks.

In practice, ARB and ARGs may be more widespread in aquacultures because most studies analyze resistance with only cultured-based methods (Guardabassi et al., 2000; Schmidt et al., 2000). Culture-independent methods, including measurements of gene copy numbers by quantitative polymerase chain reaction (qPCR), provide a less biased estimation of ARGs in the environment. The number of ARGs in marine aquacultures has been investigated by very few studies (Tamminen et al., 2011; Gao et al., 2012; Di Cesare et al., 2013; Muziasari et al., 2014), and it has never been analyzed in freshwater fish farms.

Tetracyclines are widely used in aquaculture, in particular to control furunculosis in salmonids, and oxytetracycline is a drug of first choice in the Polish fish farming industry (www.wetgiv.gov.pl). However, the continued widespread use of antibiotics has led to the development of tetracycline-resistant microorganisms in all farming operations, from fish feed (Kerry et al., 1995) to water that circulates in and out of the pond (Miranda et al., 2003). Our previous studies (Harnisz et al., 2011; Harnisz, 2013) demonstrated that tetracycline-resistant (*tet*^{R}) bacteria are sensitive to changes in the physicochemical parameters of water and antibiotic concentrations and that they are more abundant in polluted environments. Similar results were reported by Chen et al. (2013a) who confirmed that water contamination levels can be analyzed based on tetracycline resistance genes.

The aim of this study was to assess the impact of a freshwater fish farm on an antibiotic-resistant bacterial community based on the prevalence of tetracycline-resistant bacteria (TRB) and tetracycline resistance (*tet*) genes. Two parallel approaches were used to examine the prevalence of TRB and *tet* genes: a culture-based method involving standard PCR, and a method relying on quantitative PCR. To our knowledge, this paper makes the first attempt to investigate antibacterial resistance in freshwater fish farms of Central-Eastern Europe with the use of qPCR.

2. Materials and methods

2.1. Sampling sites and sampling protocol

The Drwęca River is 207.2 km long, and it drains a catchment basin with an area of 5693 km^2. The river is part of the Natura 2000 network (European Union network of nature protection areas – the aim of the network is to guarantee long-term survival of Europe's most valuable and threatened species and habitats). In the upstream section of the Drwęca River, the major sources of point pollution are household effluents and post-production water evacuated from three fish farms.

The effect of post-production water from fish farms on antibiotic-resistant bacteria in river water was analyzed in our previous study (Harnisz et al., 2011). No statistically significant differences in ARB counts were observed in samples collected at the sites located in a river stretch from the source to one of the analyzed fish farms, which emerged as a major source of tetracycline-resistant microorganisms. In view of the above, the same fish farm was selected for this experiment that relied on standard PCR and qPCR.

The farm's average annual output is estimated at 50 tons of trout. Culture ponds in the farm are supplied with water from the Drwęca River. Post-production waters are directed to two reed beds for purification, and treated effluents are discharged to the Drwęca River.

Samples of post-production water (before and after treatment in reed beds) and river water (upstream and downstream from the discharge point of treated post-production water) were collected for the study. Samples of post-production water (PPW) were collected in a canal connecting fish ponds and reed beds. Samples of upstream river water (URW) and downstream river water (DRW) were collected approximately 200 m from the discharge point of treated post-production water (TPPW) (N53°36'24.7", E20°07'33.0"). The samples were obtained in October and December 2010 and in January, March, May, July, August and October 2011. They were collected in sterile bottles, transported to the laboratory at the temperature of 4 °C and processed on the day of collection.

2.2. Culture-dependent method

2.2.1. Heterotrophic plate counts and ARB counts

URW, PPW, TPPW and DRW samples were passed through a cellulose filter (pore diameter 0.45 μm, Millipore) or diluted with saline water to obtain 30–300 colony forming units (CFU) per plate. Heterotrophic plate counts (HPC) were determined on plates containing the TSA medium (Oxoid Ltd.). The plates were incubated at 28 °C for 24 h. The size of bacterial populations resistant to tetracycline (oxytetracycline and doxycycline) was determined on plates containing the TSA medium with the addition of antibiotics (Sigma). Antimicrobial doses were determined at 16 mg L^{-1} in accordance with the guidelines of the Clinical and Laboratory Standard Institute (CLSI, 2010). Resistant microorganisms were incubated at 28 °C for 24 h.

Short incubation time could support the growth of bacterial species that are easy to culture, however, the time and temperature of incubation were chosen based on our previous experiment that involved bacterial cultures isolated from the Drwęca River (Harnisz et al., 2011). In the said study, we observed that prolonged incubation (to 48–72 h) led to the proliferation of bacteria that were not resistant to the antibiotic added to the TSA medium. The above can probably be attributed to slow antibiotic degradation during incubation. Longer incubation time resulted in rapid growth of molds, which prevented the isolation of resistant bacteria. In the same experiment, bacteria resistant to tetracycline were incubated at 14 °C and 28 °C, but a statistical analysis of the abundance of Tet^R 14 °C and Tet^R 28 °C bacteria revealed significant differences only in the size of Tet^R 28 °C populations ($p = 0.0011$). Based on the above results, bacteria were incubated at 28 °C for 24 h in the present study.

2.2.2. Minimum inhibitory concentrations and antibiotic susceptibility testing

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^{-1}.

OTC^R and DOX^R isolates were subjected to sensitivity tests against 12 antimicrobials from seven classes: (1) penicillins: amoxicillin/clavulanic acid (AMC 75 μg), mezlocillin (MEZ 75 μg), piperacillin (PRL 75 μg); (2) cephalosporins: ceftazidime (CAZ 30 μg), cefotaxime (CTX 30 μg); (3) glycylcyclines: tigecycline (TGC 15 μg); (4) fluoroquinolones: ciprofloxacin (CIP 5 μg), enrofloxacin (ENR 5 μg), norfloxacin (NOR 10 μg); (5) aminoglycosides: tobramycin (TOB 10 μg); (6) trimethoprim/sulfamethoxazole (SXT 1.25/23.75 μg) and (7) tetracyclines: tetracycline (TE 30 μg), All disks were supplied by Oxoid. Resistance was estimated by measuring the growth inhibition zone according to CLSI guidelines (2010).

2.2.3. Identification of isolates and determination of ARGs

Bacterial DNA templates were prepared by boiling or CTAB procedures (Harnisz et al., 2015).