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Microbiological characterization of aquatic microbiomes targeting taxonomical marker genes and antibiotic resistance genes of opportunistic bacteria

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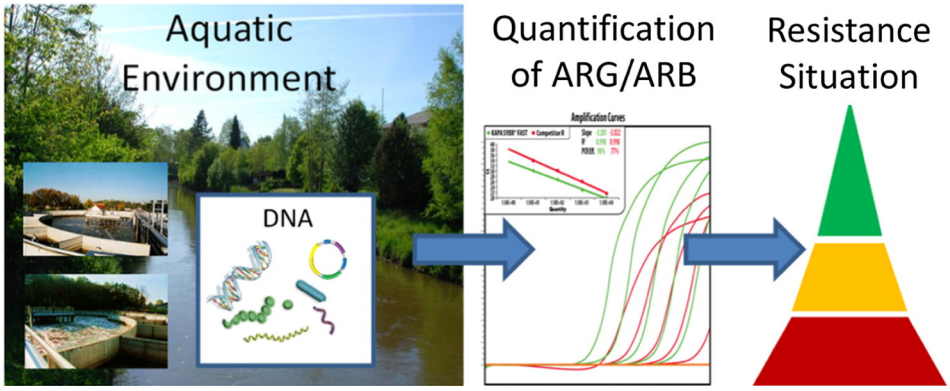
HIGHLIGHTS

- We investigated the abundances of ARGs and opportunistic bacteria in aquatic systems
- Molecular biological methods were applied to analyze bacterial populations.
- Specific ARG were more abundant than the corresponding bacteria
- Volume-based references might underestimate the abundances of ARGs and opportunistic bacteria
- Chemical analyses were run to study the presence of antibiotics

GRAPHICAL ABSTRACT



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ABSTRACT

The dissemination of medically relevant antibiotic resistance genes (ARGs) (*bla*VIM-1, *van*A, *amp*C, *erm*B, and *mec*A) and opportunistic bacteria (*Enterococcus faecium/faecalis*, *Pseudomonas aeruginosa*, *Enterobacteriaceae*, *Staphylococcus aureus*, and CNS) was determined in different anthropogenically influenced aquatic habitats in a selected region of Germany. Over a period of two years, four differently sized wastewater treatment plants (WWTPs) with and without clinical influence, three surface waters, four rain overflow basins, and three groundwater sites were analyzed by quantitative Polymerase Chain Reaction (qPCR). Results were calculated in cell equivalents per 100 ng of total DNA extracted from water samples and per 100 mL sample volume, which seems to underestimate the abundance of antibiotic resistance and opportunistic bacteria. High abundances of opportunistic bacteria and ARG were quantified in clinical wastewaters and influents of the adjacent WWTP. The removal capacities of WWTP were up to 99% for some, but not all investigated bacteria. The abundances of most ARG targets were found to be increased in the bacterial population after conventional wastewater treatment. As a consequence, downstream surface water and also some groundwater compartments displayed high abundances of all four ARGs. It became obvious that the dynamics of the ARG differed from the fate of the opportunistic bacteria. This underlines the necessity of an advanced microbial characterization of anthropogenically influenced environments.

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1. Introduction

Surveillance of antimicrobial drug resistance in aquatic systems is of increasing importance due to the high usage of antibiotics in human and veterinary medicine as well as in agricultures (Ashbolt et al., 2013; Hirsch et al., 1999; Lupo et al., 2012). The WHO describes this development to be a global problem of emerging concern (Cadman, 2014). For this reason, the identification and monitoring of critical points in aquatic systems, which provide for and enhance the transfer and dissemination of antibiotic resistance genes, are of great importance (Marti et al., 2014b). Germany used more than 1619 tons of antibiotics for veterinary purposes in 2012 (Federal Office of Consumer Protection and Food Safety, BVL), (Meyer et al., 2013). In addition, Germany counted about 38 million prescriptions of antibiotics in human medicine for 684 million Euros in 2011 (Altiner et al., 2012). This represents one of the top averages of antibiotic consumption in the European countries. Due to the high application, incorrect handling, and incomplete metabolism, antibiotics can be found in wastewater and aquatic habitats influenced by wastewater. The concentrations of pharmaceuticals found in wastewater are far below concentrations used in hospital settings, but are known to induce molecular responses and are involved in the evolution of antibiotic-resistant bacteria (Davies, 2006). Five major mechanisms are known to contribute to the antibiotic resistance development and dissemination in the environment: Horizontal gene transfer of antibiotic resistance genes among bacteria (Fall et al., 2007), selective pressure of antimicrobial substances (even in low concentrations) and heavy metals (Davies et al., 2006; Goh et al., 2002; Hirsch et al., 1999; Seiler and Berendonk, 2012), predispositions for genetic mutations and recombination events, and the dissemination of antibiotic-resistant bacteria from human and veterinary medicine (Cantas et al., 2013). In addition, phages can act as transfer-vehicles for ARG (transduction) and are widely distributed in the environment (Balcazar, 2014). These mechanisms contribute to the antibiotic resistance pattern by a direct selection of molecular processes and by spontaneous genetic events to adapt to the changing environment. The definition of antibiotic-resistant bacteria is based on breakpoints (EUCAST/Clinical and Laboratory Standard Institute) used to determine the outcome of the antibiotic treatment of patients. It may be questioned, however, whether these breakpoints are suitable for the characterization of environmental bacteria. Alternative approaches might be needed for an adequate microbial characterization of aquatic habitats. In addition, the genetic information of antibiotic resistance genes is not limited to specific bacteria, but can be transferred via mobile genetic elements (plasmid, transposon, integron) to different species (horizontal gene transfer) (Ashbolt et al., 2013; Jechalke et al., 2013; Martínez, 2008) These mechanisms contribute to the antibiotic resistance pattern in aquatic habitats. This aspect is also important with regard to raw water used for drinking water processes when medically relevant genes from pathogens or opportunistic bacteria are transferred to non-pathogenic bacteria. To date, hardly any comprehensive environmental data have been made available to develop and make a microbiological risk assessment based on the occurrence of antibiotic resistance found in the environment. The European Water Framework Directive is dedicated to ensuring an adequate good quality status of all water bodies according to specific environmental quality standards, but it does not include clinically relevant antibiotic resistance (European Community Directive 2000/60/EC).

In view of the continuously discharging of antibiotic resistance in aquatic habitats, this study used a molecular approach that bypasses the limitations of cultivation methods used in e.g. hospitals to detect and quantify genes and gene carriers of clinical significance. Molecular analyses allow for the microbiological characterization of different aquatic habitats to assess the dissemination of ARG and opportunistic bacteria in natural populations and to identify and monitor critical water systems and potential microbiological risks for human health.

2. Experimental selection

2.1. Sampling sites

Over a period of two years, 16 sampling sites in a selected region of Germany were investigated to determine abundances of opportunistic bacteria and ARG. 24-hour samples of clinical wastewater, in- and effluents of WWTP as well as water samples of receiving bodies (rivers), groundwater, and rain overflow basins were collected and analyzed by molecular biology methods (Fig. 1).

The clinical wastewater was sampled from the first accessible sewer entrance. Sampling site S1 contains wastewater from a hospital with 1000 beds, a nursery home, and a military hospital. The corresponding WWTP processes wastewater from a city with 445,000 inhabitants (sampling sites $S3_{in}$ and $S4_{out}$) and is equipped with a conventional three-treatment process (nitrification, denitrification, phosphorus elimination).

The second municipal WWTP with the sampling sites $S5_{in}$ and $S6_{out}$ is located in a smaller city with 16,600 inhabitants. Here, a much smaller hospital with 80 beds (S2) discharges wastewater to the above-mentioned municipal WWTP. Two additional WWTPs were under investigation, both equipped with a tertiary treatment. One of the two WWTPs processes wastewater of 16,000 inhabitants (sampling sites $S7_{in}$ and $S8_{out}$), and the other WWTP cleans wastewater of 2500 inhabitants (sampling sites $S9_{in}$ and $S10_{out}$).

The sampling sites S11, S12, and S13 represent surface water systems, with S11 being positioned on the river Danube, whereas S12 and S13 represent sampling sites on two feeder rivers.

Four rain overflow basins were sampled to investigate the possible influence of runoffs from agricultural and residential areas on the bacterial and ARG pattern (S14, S15, S16, and S17).

To investigate potential contamination of the aquifer by a disposal site, two groundwater sampling sites were analyzed (S18 and S19). The third groundwater sampling site was investigated for contamination by percolation of river 2 (S20).

2.2. Sample preparation

Water samples were collected in sterile glass bottles and stored in the dark at 4 °C. Native water samples were prepared for DNA extraction within 24 h. Depending on turbidity, wastewater samples were filtered through a 45 mm polycarbonate membrane with 0.2 μm pore size (Whatman nucleopore membrane, Sigma, Munich, Germany). Up to 6 L of groundwater was filtered for biomass enrichment. In addition, up to 0.5 L was used for DNA extraction from surface waters, rain overflow basins, and WWTP effluents. A volume of 0.2 L of each clinical wastewater and WWTP influent was centrifuged at 20,400×*g* for 15 min (Avanti J-25, Fullerton, California) and resuspended in 1 mL sterile water. DNA extraction was performed using the FastDNA Spin kit for soil (MP Biomedical, Illkrich, France) using the lysing matrix E and the manufacturer's protocol for wastewater. To enhance the extraction efficiency, two steps of elution with 50 μL sterile water were performed. For DNA extraction from highly turbid wastewater, suspended samples were treated with 40 mg of polyvinylpolypyrrolidone (Sigma, Munich, Germany) in 1.6 mL of ASL stool lysis buffer from the extraction kit. After heating the samples at 95 °C for 10 min, the supernatants were treated with a PCR inhibitor absorbent matrix (Inhibit-ex, QIAamp DNA Stool Mini Kit, Qiagen, Hilden, Germany). After centrifugation, a QiaAmp Mini kit for DNA extraction from suspended water samples of high-turbidity wastewater was used. After precipitation, a proteinase K treatment was applied for treating the supernatant. To enhance the DNA extraction efficiency, the final elution volume was reduced to 50 μL and the incubation time was increased to 5 min. The process was repeated twice. The quantities and purities of the DNA extracts were measured by the NanoDrop ND-1000 instrument (Peqlab Biotechnology, Erlangen, Germany).