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Does human activity impact the natural antibiotic resistance background? Abundance of antibiotic resistance genes in 21 Swiss lakes

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ABSTRACT

Antibiotic resistance genes (ARGs) are emerging environmental contaminants, known to be continuously discharged into the aquatic environment via human and animal waste. Freshwater aquatic environments represent potential reservoirs for ARG and potentially allow sewage-derived ARG to persist and spread in the environment. This may create increased opportunities for an eventual contact with, and gene transfer to, human and animal pathogens via the food chain or drinking water. However, assessment of this risk requires a better understanding of the level and variability of the natural resistance background and the extent of the human impact. We have analyzed water samples from 21 Swiss lakes, taken at sampling points that were not under the direct influence of local contamination sources and analyzed the relative abundance of ARG using quantitative real-time PCR. Copy numbers of genes mediating resistance to three different broad-spectrum antibiotic classes (sulfonamides: *sul1*, *sul2*, tetracyclines: tet(B), tet(M), tet(W) and fluoroquinolones: *qnrA*) were normalized to copy numbers of bacterial 16S rRNA genes. We used multiple linear regression to assess if ARG abundance is related to human activities in the catchment, microbial community composition and the eutrophication status of the lakes. *Sul* genes were detected in all sampled lakes, whereas only four lakes contained quantifiable numbers of *tet* genes, and *qnrA* remained below detection in all lakes. Our data indicate higher abundance of *sul1* in lakes with increasing number and capacity of wastewater treatment plants (WWTPs) in the catchment. *sul2* abundance was rather related to long water residence times and eutrophication status. Our study demonstrates the potential of freshwater lakes to preserve antibiotic resistance genes, and provides a reference for ARG abundance from lake systems with low human impact as a baseline for assessing ARG contamination in lake water.

1. Introduction

Ever since the introduction of antibiotics into clinics in the 1940s (heralding the beginning of the modern antibiotic era), immense and often imprudent use of antibiotics in human and veterinary settings has selected for resistance and multiresistance against antibiotics in pathogens and commensal bacteria (Baquero and Blázquez, 1997; Davies and Davies, 2010). The antibiotic resistance genes (ARGs) that confer these resistance traits are currently discussed as emerging environmental contaminants (Pruden et al., 2006; Rysz and Alvarez, 2004). Major sources of pollution with ARG are human and animal-derived wastewaters or feces and manure, respectively, entering the environment via wastewater treatment plants (WWTPs) or direct application to soil (Czekalski et al., 2012; Graham et al., 2010; Heuer et al., 2008; Heuer and Smalla, 2007; Rizzo et al., 2013; Zhang et al., 2009). On the other hand, antibiotics are naturally produced by environmental microbiota, though in concentrations much lower as compared to those used in antibiotic therapy (Aminov, 2009; Davies et al., 2006). Similarly, ARG are naturally present in environmental bacteria (Aminov, 2010; D'Costa et al., 2006). Although defense against competitors and resistance to natural antibiotics may play a role, it has become increasingly apparent that the role of antibiotics and ARG in environmental bacteria is often different from the "weapon and shield" function we observe in clinics (Alonso et al., 2001; Aminov, 2009; Martinez, 2008). It has been documented, that many ARG were originally localized on the chromosome of harmless bacteria; but since the beginning of the modern antibiotic era ARG are increasingly found on mobile genetic elements in pathogens and fecal bacteria (Datta and Hughes, 1983; Gillings et al., 2008). Thus, rapid dissemination of ARG via horizontal gene transfer was facilitated. Vice versa, ARG located on mobile elements in human and animal derived pathogens might easily be exchanged with environmental microbiota, e.g., during sewage treatment or discharge of sewage into the receiving water bodies (Baquero et al.,

Abbreviations: ARG, Antibiotic resistance gene; WWTP, Wastewater treatment plant; NW, number of WWTPs; CW, capacity of WWTPs given as inhabitant equivalents; NH, number of hospitals; CH, capacity of hospitals given as person occupancy per day; CA, catchment area; TP, total phosphorous; RT, retention time; NC, number of cattle; UA, urban areas; NO, nitrate; LV, lake volume; AA, agricultural areas; NP, number of pigs; Stdev, standard deviation of mean.

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2008). As ARG are often co-selected by non-antibiotic pollutants, such as heavy metals or other biocides, high amounts of ARG are likely to persist in the environment, which may have consequences for the functionality of the microbiosphere and increases the level of the natural ARG background (Martinez, 2009). Thus a vicious circle might be created by which ARG can be recycled faster and faster between bacteria in natural and clinical settings.

Freshwater bodies harbor natural assemblages of bacteria that may allow sewage-derived ARG (and the mobile genetic elements they reside on) to persist and eventually return to human and animal pathogens, as often the same water body may serve as receiving water for wastewater and as drinking water reservoir (Baquero et al., 2008). Though the potential of freshwater bodies to preserve ARG has been proposed more than two decades ago (Jones et al., 1986), our understanding of this function is still in its infancy. Many studies aiming to document antibiotic resistance pollution have applied culture-based single-indicator-strain approaches (Picao et al., 2008; Roberts et al., 2009; Suzuki et al., 2013; West et al., 2010; Zhang et al., 2009; Zurfluh et al., 2013), which do not capture large proportions of natural freshwater microbial communities. Recent studies have increasingly applied molecular tools, such as PCR and qPCR (Cummings et al., 2010; Heuer et al., 2008; Mao et al., 2013; Marti and Balcázar, 2013; Marti et al., 2013; Muziasari et al., 2014; Stalder et al., 2014; Suzuki et al., 2013; Walsh et al., 2011), or metagenomics, e.g., for investigating links between anthropogenic wastewater discharge and contamination of a marine environment (Port et al., 2012). Among freshwater systems, rivers have received the most attention (Graham et al., 2010; Pei et al., 2006; Storteboom et al., 2010), while research on freshwater lakes, particularly using molecular techniques, is comparatively scarce (Auerbach et al., 2007; Czekalski et al., 2012). Rivers transport discharged pollutants away from the site of contamination rapidly, whereas the residence time of water in lakes, which is linked to retention time of contaminants (irrespective of vertical transport, e.g., sedimentation rates) is much longer (Oliviera, 2007). Thus, lakes have the potential to store and accumulate ARG to a greater extent than rivers. This highlights the importance of conducting further research on the resistance loads in lakes and reservoirs.

Switzerland is a country with comparatively low human antibiotic consumption (Kronenberg et al., 2006) and average consumption in the veterinary sector (European Medicines Agency, 2011). Switzerland has many freshwater lakes and watersheds that are fed by meltwater and are naturally poor in nutrients (oligotrophic). As in many other places, Swiss lakes were affected by anthropogenic eutrophication (Bigler et al., 2007). Major causes of eutrophication are partly the same as for ARG contamination: discharged sewage and animal waste, but fertilization of crops is also involved. Extensive measures were taken in Switzerland to return lakes to their natural trophic status, e.g., by limiting P-and N-discharge from sewage by building WWTPs and a ban of phosphates in detergents (Vonlanthen et al., 2012). Nevertheless, we assumed that the eutrophication state of lakes remains a good indicator for the intensity of human impact and that WWTPs, in spite of their effectiveness in reducing nutrients and bacteria in sewage, discharge important amounts of ARG into natural water bodies. In the present study we aimed to explore the relative abundance of ARG in 21 freshwater lakes in Switzerland. Abundance of genes mediating resistance to three different broad-spectrum antibiotics (sulfonamides: *sul1*, *sul2*, tetracyclines: tet(B), tet(M), tet(W) and fluoroquinolones, *qnrA*), was determined using quantitative real-time PCR. These compounds are used in different proportions in human and veterinary applications: Sulfonamide use is 4 times higher in veterinary compared to clinical settings (Stoob, 2005) and tetracyclines are likewise mostly applied in animal husbandry (Büttner et al., 2011). In contrast, fluoroquinolones are of greater importance in human medicine (Plüss-Suard et al., 2011).

We hypothesized that ARG abundance in lakes can be linked to the intensity of human activities in the lake's catchment. Further, we hypothesized that, due to elevated use of antibiotics in veterinary as compared to human medicine (Czekalski, 2013; Kovalova et al., 2011; Moulin et al., 2008; Stoob, 2005), agricultural activities, especially animal farming, have a stronger impact on ARG pollution than urban activities and contamination sources, such as WWTPs. Based on the idea that elevated nutrient contents in lakes can result from both urban and agricultural drainage, we expected increased levels in P- and N-content to be accompanied by increased ARG levels in lakes, due to similar contamination sources. Composition of the prevailing microbial communities in the lakes was also considered as a potential explanatory variable for ARG abundance. In addition, we aimed at measuring the variability of ARG abundance among the lakes least impacted by human inputs to establish a baseline for the background level of ARG abundance for lake water in the study area.

2. Materials and methods

2.1. Study sites and sampling campaigns

A total of 21 Swiss lakes (Fig. 1) varying in size, trophic status, geographic location and land use in the catchment area were sampled between July and October 2011, except for Lake Brienz, which was sampled in May 2009. Each lake was sampled once at its deepest point by taking an integrated sample over the upper 5 m using a 5 m plastic tube. The water was filled into pre-sterilized 5- or 10-l water cans and transported to the laboratory for filtration within 1 day. Between 3 and 6 l of each lake sample was at first pre-filtered through 3-μm-pore size Isopore polycarbonate filters (Ø 142 mm, Merck Millipore, Billerica, MA, USA) for removal of larger organisms and particles. Subsequently, pre-filtered water was filtered onto 0.2 μm pore-size polycarbonate membrane filters (Ø 142 mm, Millipore). Filters were stored at −80 °C until DNA extraction.

2.2. DNA extraction

DNA was extracted from microorganisms filtered from lake water according to a protocol modified from Fuhrman et al. (1988). Clean filters were processed to obtain extraction blanks. Each filter was cut into quarters. Three quarters were processed individually as extraction replicates. Filter quarters were cut into small wedges and disaggregation of cells from the filters and disruption of cell envelopes were carried out in two steps: First, 2 glass beads of 5 mm diameter (Sigma Aldrich, St. Gallen, Switzerland) and 1.2 ml of STE buffer (Fuhrman et al., 1988) were added and processed for 20 s at 4 ms^{-1} in a FastPrep-24 bead-beating system (MP Biomedicals, Santa Ana, CA, USA). Secondly, 50 μl of 20% sodium dodecyl sulfate solution was added and tubes were placed into a boiling water bath for 2 min. DNA was extracted with 400 μl each of phenol (pH 8) and chloroform/isoamyl alcohol (24:1), followed by 2× re-extraction of the supernatant with 800 μl of CIA. DNA was precipitated with 600 μl of isopropanol for 30 min on ice and then centrifuged for 30 min at 4 °C. The DNA pellet was washed with 500 μl of 70% ice-cold ethanol. After ethanol removal, DNA pellets were dried in a Speed Vacuum Concentrator (Eppendorf AG, Hamburg, Germany), and resuspended in Tris-EDTA buffer (pH 7.4). All chemicals used for extraction were purchased from Sigma Aldrich. DNA-extracts were checked for the presence of DNA bands by agarose gel electrophoresis (1% Agarose, run for 45 min at 80 V). Moreover, DNA concentration of extracts was determined in order to check for sufficient DNA-yield for subsequent analysis, using the Quant-iT PicoGreen® DNA quantification kit (Invitrogen, Basel, Switzerland). DNA extracts were stored at −20 °C until use in qPCR assays.

2.3. qPCR assays

DNA extracts were screened for abundance of five different antibiotic resistance genes (*sul1*, *sul2*, tet(B), tet(M), tet(W) and *qnrA*) using quantitative real-time PCR. Additionally, bacterial 16S rRNA gene fragments were quantified for normalizing resistance gene copy numbers