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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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A B S T R A C T

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.
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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

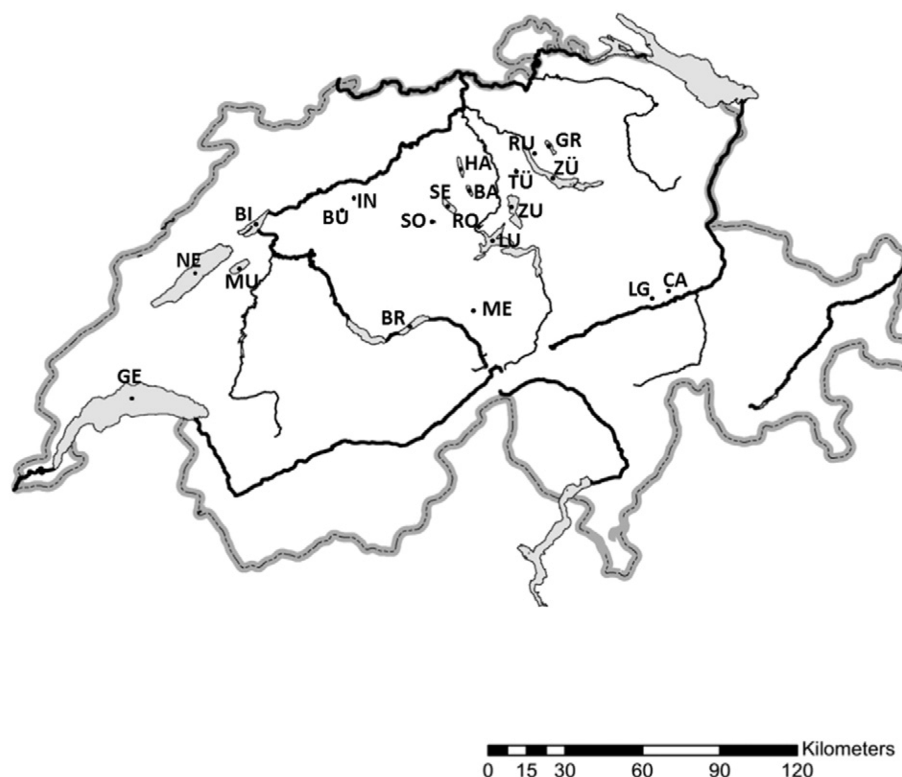

Fig. 1. Map indicating the geographic location of 21 Swiss Lakes sampled for our survey (Abbreviations of lake names are explained in Table 1. The map was created with ArcGIS (ESRI®-ArcMap 10, Redlands, CA). 1.5-Column fitting image.

per sample to the bacterial population size of each lake sample. Standard dilutions of plasmids containing the respective target genes were prepared in a range of 30 to 3×10^6 copies per 5 μl as described previously (Czekalski et al., 2012).

qnrA was amplified using QuantiTect SYBR Green PCR chemistry (Qiagen, Hombrechtikon, Switzerland). The remaining resistance genes and 16S rRNA genes were amplified using the TaqMan® Environmental Master Mix 2.0 (Life Technologies Corp., Applied Biosystems, Carlsbad, CA, USA). All qPCR assays contained the respective PCR buffer, primers, probe and $MgCl_2$ concentrations as described in the respective publications (Cummings et al., 2010; Heuer et al., 2008; Smith et al., 2006; Suzuki et al., 2000; Walsh et al., 2011), 5 μl of template or standard dilution, and water to give a final reaction volume of 25 μl. Each standard dilution was analyzed in triplicate, whereas each of the triplicate extracts of samples and extraction blanks was analyzed only once. In order to minimize effects of PCR inhibitors and to assure that all samples fell within the linear range of the standard curves, DNA extracts were diluted 1:10 for analysis of resistance genes and 1:500 for 16S rRNA gene assays. Triplicates of 10 ng μl^{-1} of salmon sperm DNA and nuclease free water were analyzed in each assay as no template control and PCR blank, respectively. qPCR assays were performed in 96 well plates in a 7500 Fast Real-Time PCR System (Applied Biosystems) and data analysis was carried out using default settings of the 7500 software (v.2.06, Applied Biosystems).

Samples were considered as not quantifiable for a target gene if the standard deviation (SD) of Ct values of the filter triplicates was greater than 0.5 (unquantifiable sample) OR if the mean Ct value of the triplicates was equal to or greater than the mean Ct value of the first standard dilution with a SD of Ct values greater than 0.5 (below limit of quantitation). Samples were considered as below detection if their mean Ct value was greater than that of the lowest Ct value obtained from any negative controls for this assay OR if for 2 or more out of 3 Ct values of the replicates were not recorded (Czekalski et al., 2014). Limits of quantitation and detection for each qPCR assay are summarized in Table A.1 of Appendix A).

2.4. Automated Ribosomal Intergenic Spacer Analysis (ARISA)

ARISA-PCR conditions and fragment analysis of PCR products followed the procedure described by Yannarell et al. (2003) with some changes (Bürgmann et al., 2011). Data generated from sequencing were analyzed in Gene Mapper® Software version 4.0 (Life Technologies Corp., Applied Biosystems, Carlsbad, CA). Peaks with sizes between 350 and 1250 bp and a minimum peak height of 75 fluorescence units were taken into account. Prior to statistical analysis of sequencing results, the ARISA peaks were properly binned by applying the automated and interactive binner algorithms (Ramette, 2009) using R software (version 2.14.2; R Core Team, 2012). A window size of 2.5 bp was used for binning.

2.5. Environmental data on lakes and catchment land-use

General lake parameters and information on their nutrient content are presented in Table 1. Based on their total phosphorous (TP) content lakes were assigned to trophic classes (data for TP and total nitrogen (NO) were obtained from routine measurements of Swiss environmental authorities, references are given in the header of Table 1). As most alpine and pre-alpine lakes were naturally in an oligotrophic to mesotrophic state prior to the industrial era, eutrophic and hyper-eutrophic lakes result from human impact in the lake's catchment (Bigler et al., 2007). The catchment area of each lake was determined from a landscape model of the hydrographical units of Switzerland provided by BAFU (2012) in ArcGIS (ESRI®ArcMap 10, Redlands, CA). Information on the land use pattern in the catchment area of each lake were obtained in ArcGIS from data provided by the Swiss Federal Statistical office (summarized in Table 2). The dataset includes general catchment characteristics, such as proportions of urban and agricultural land use in each lake's catchment. Moreover, specific data were obtained for counts of farm animals (pigs and cattle) and number and capacity of sewage treatment plants and hospitals in each catchment area, all of which are considered potential contamination sources of antibiotic resistance genes that may affect the lakes.

Table 1
Names of sampled lakes, their abbreviations used in figures, as well as general lake characteristics, nutrient content, assigned trophic status and geographical coordinates. Units given in (). Lake volume, total phosphorous and nitrate content were used as explanatory variables in stepwise linear regression models, abbreviations for these variables used in statistical models are given in (). References for data acquisition on the listed parameters for each lake are given in the footer of this table.

Name of lake	Lake abb.	Surface area (km^2)	Max depth (m)	Lake volume (LV) (10^6 m^3)	Lake water retention time (RT) (a)	Total phosphorous (TP) ($\mu g\ l^{-1}$)	Total nitrate (NO) ($mg\ l^{-1}$)	Trophic status	Longitude	Latitude
Lake Baldegger	BA[a,f]	5.2	66	173	4.20	28	1.6	Eutrophic	8.261	47.199
Lake Biel	BI[c]	37.2	74	1120	0.16	18	1.4	Mesotropic	7.174	47.086
Lake Brienz	BR[d,f]	30	261	5170	2.69	3	0.36	Oligotrophic	7.966	46.728
Lake Burgäschi	BU[b,e]	0.23	31	2.5	NA	99	2	Hypereutrophic	7.668	47.169
Lake Cauma	CA[a,e]	0.1	30	0.65	NA	7.2	NA	Oligotrophic	9.296	46.82
Lake Geneva	GE[a]	580	309	89,000	11.40	22	0.4	Mesotropic	6.592	46.456
Lake Greifen	GR[a]	8	32	148	1.12	50	1.5	Eutrophic	8.677	47.353
Lake Halwil	HA[a,d]	30	261	280	3.90	45	0.77	Eutrophic	8.211	47.297
Lake Inkwil	IN[a,b,e]	0.12	6	215	NA	152	NA	Hypereutrophic	7.662	47.198
Lake Grond	LG[b]	0.05	5	0.03	NA	20	NA	Mesotropic	9.257	46.808
Lake Lucerne	LUu[e,f]	114	114	11,900	3.40	5	0.6	Oligotrophic	8.577	47.293
Lake Melch	ME[a]	0.54	18	4	NA	15.9	NA	Mesotropic	8.273	46.773
Lake Murten	MU[c,f]	22.7	45	550	1.60	24	2.1	Mesotropic	7.085	46.932
Lake Neuchatel	NE[c]	218	152	13,900	8.20	9	1.2	Oligotrophic	6.859	46.906
Lake Rot	RO[a,f]	0.46	16	3.8	0.44	25	0.1	Eutrophic	8.185	47.041
Lake Rumen	RU[b]	0.02	2	0.014	NA	50	0.36	Eutrophic	8.353	47.194
Lake Sempach	SE[a,f]	14.4	84	660	16.90	20	0.55	Mesotropic	8.152	47.144
Lake Soppen	SO[a,e]	0.236	27	2.9	3.20	372	0.775	Hypereutrophic	8.081	47.09
Lake Türler	TU[d,f]	0.497	22	6.5	2.00	15	0.5	Mesotropic	8.503	47.27
Lake Zürich	ZS[a]	68	136	3300	1.21	20	0.6	Mesotropic	8.401	47.019
Lake Zug	ZU[d]	38	198	3174	14.70	94	0.3	Eutrophic	8.483	47.161

NA: no data acquired.
[a] BAFU, "Wasserqualität Seen",http://www.bafu.admin.ch/gewaesserschutz/01267/01269/01271/index.html?lang=de.
[b] European Diatom Database (http://cratacula.ncl.ac.uk/Eddi/jsp/index.jsp, data from 1993–1998).
[c] Die 3 Seen (www.die3seen.ch).
[d] Keller et al. (2008).
[e] Wikipedia (https://de.wikipedia.org/wiki/Wikipedia:Hauptseite).
[f] Cantonal monitoring data 2011 provided by Eawag.

2.6. Statistics

Resistance gene copy numbers of each sample were normalized to bacterial 16S rRNA gene copy numbers of the same sample and are reported as indicators of the relative abundance of resistance genes within the bacterial population in each lake.

Two-sample *t*-tests (assuming unequal variances) and calculation of quartiles were performed in order to reveal statistically significant differences of ARG-levels between the studied lakes and to identify outliers. Correlation analysis between different antibiotic resistance genes (ARG) as well as between ARG and lake and catchment data were performed in order to evaluate similar distribution patterns of ARG and to identify important catchment properties linked to increased ARG abundances in lakes. Prior to analysis, variables were either log-transformed (Table 1: lake volume (LV), total phosphorous (TP), total nitrate (NO), lake water retention time (RT), Table 2: number of WWTPs (NW), hospitals (NH), pigs (NP), cattle (NC) and pigs and cattle together (NP + NC), capacity of WWTPS (CW) and hospitals (CH)) or

Table 2
Names of sampled lakes, their abbreviations used in figures, and land use parameters for each lake catchment, which were used as explanatory variables in stepwise linear regression models, their model abbreviations or units are given in (): number (NW) and capacity (CW) of wastewater treatment plants (given as inhabitant equivalents), number (NH) and capacity of hospitals (given as persons occupancy per day), number of cattle (NC) and pigs (NP) present in the catchment area (CA) of each lake. Proportional (%) information on land use patterns in lake catchments is given in columns 10 and 11, information on the presence or absence of hospitals and wastewater treatment plants is given in the last column.

Name of lake	Lake abb.	Catchment area (CA) (km^2)	Number WWTPs (NW)	Capacity WWTPs (CW)	Number hospitals (NH)	Capacity hospitals (CH)	Number cattle (NC)	Number pigs (NP)	% urban area (UA)	% agricultural area (AA)	WWTPs & hospitals yes/no
Lake Baldegger	BA	73.2	1	11,535	0	0	10,106	29,287	5.6	72.3	Yes
Lake Biel	BI	8235.5	162	1,060,847	29	1,607,427	387,640	186,348	4.6	26.5	Yes
Lake Brienz	BR	1137.4	10	20,038	6	481,527	12,182	886	1.3	1.1	Yes
Lake Geneva	GE	7766.1	124	777,131	34	2,158,508	72,974	16,244	5.0	7.3	Yes
Lake Greifen	GR	163.8	8	112,273	3	144,093	11,949	3619	17.3	51.8	Yes
Lake Halwil	HA	139.3	2	18,962	0	0	17,340	44,366	9.5	62.2	Yes
Lake Lucerne	LU	2242.7	22	145,192	17	1,088,704	60,187	41,616	2.9	1.3	Yes
Lake Murten	MU	711.3	30	86,277	2	28,375	55,848	20,479	5.8	67.4	Yes
Lake Neuchatel	NE	2677.6	102	310,306	6	108,234	126,701	50,405	4.9	42.6	Yes
Lake Sempach	SE	75.4	1	9282	1	46,777	8198	29,964	6.6	62.5	Yes
Lake Zürich	ZS	1825.8	30	355,917	18	497,899	55,071	38,562	7.1	10.5	Yes
Lake Burgäschi	BU	5.4	0	0	0	0	651	1607	9.2	56.9	No
Lake Cauma	CA	4.0	0	0	0	0	44	0	2.2	0.0	No
Lake Inkwil	IN	4.7	0	0	0	0	357	633	11.9	67.0	No
Lake Grond	LG	1.4	0	0	0	0	30	0	17.3	0.0	No
Lake Melch	ME	7.8	0	0	0	0	292	304	0.0	0.0	No
Lake Rot	RO	4.4	0	0	0	0	391	621	37.5	36.0	No
Lake Rumen	RU	1.6	0	0	0	0	0	0	14.2	3.7	No
Lake Soppen	SO	1.6	0	0	0	0	246	978	0.0	78.7	No
Lake Türler	TU	5.2	0	0	0	0	414	73	0.3	49.6	No
Lake Zug	ZU	250.0	0	0	0	0	17,883	18,251	9.0	21.7	No

arcsin-transformed in the case of proportions (Table 2, (%) urban (UA) and agricultural areas (AA) and ARG). In order to check whether land use variables normalized to the catchment area (assuming effects are density dependent) or to the lake volume (accounting for dilution effects) may reveal a statistical significant relation to lake ARG-levels, additional correlation analysis were performed with land use variables (NW, CW, NH, CH, NC, NP and NC + NP) normalized to either catchment size (CA) or lake volume (LV). All correlation analyses were performed using the data analysis tool in Microsoft Excel.

Stepwise multiple linear regression analyses were performed using the linear model function in the R statistical software (version 2.14.2; R Core Team, 2012). All variables (LV, TP, NO, RT (Table 1), CA, NW, CW, NH, CH, NP, NC, UA, AA (Table 2)) were checked for multicollinearity by calculating their variation inflation factors (VIFs). The variables "number of WWTPs" (NW), "number of hospitals" (NH) and "number of cattle" (NC) had to be removed due to a VIF-value greater than 10 (StataCorp, 1997). After their removal, all variables exhibited VIFs below 10, resulting in the initial models [A1,2] with response variable *sul1* and *sul2*, respectively. Additionally, same as for correlation analysis, land use variables (NW, CW, NH, CH, NC, NP and NC + NP) were normalized to either catchment size (CA) or lake volume (LV), resulting in initial models [B1,2] and [C1,2], respectively.

[A1,2] ARG[1] ~ CW + CH + NP + UA + AA + TP + NO + RT

[B1,2] ARG[1] ~ CW/CA + CH/CA + NP/CA + UA + AA + TP + NO + RT

[C1,2] ARG[1] ~ CW/LV + CH/LV + NP/LV + UA + AA + TP + NO + RT

Based on F-tests the least significant variables were removed from the model by stepwise variable reduction. Selection of the best model was based on Akaike Information Criterion (AIC) values and the strength of evidence for each model of one set was estimated from their likelihoods and Akaike weights (compare Table A.2(A–C) for *sul1* and Table A.3(A–C) for *sul2*).

Community similarity analysis was performed based on Hellinger-transformed ARISA peak area data in R version 2.14.2 (R Core Team, 2011), using the BiodiversityR and vegan packages (Oksanen et al., 2012; Kindt and Coe, 2005). Principle Coordinates Analysis (PCoA) in five dimensions was performed using the Bray distance metric. Centroid scores for each lake and each dimension were extracted with the envfit function based on 1000 permutations.

3. Results

3.1. Abundance of antibiotic resistance genes in Swiss lakes

Sulfonamide resistance genes *sul1* and *sul2* were detected in quantifiable amounts in all sampled lakes (Fig. 2(A)). The highest abundance of *sul1* ($2.1 \cdot 10^{-1}$) was found in Lake Baldegg, which was significantly different (pairwise *t*-test, $p < 0.01$) from levels found in the remaining lakes. Further, Lake Brienz, Lake Greifen, Lake Rumen, and Lake Zürich exhibited high (upper quartile) *sul1* gene abundance, ranging from $4 \cdot 10^{-2}$ (Lake Brienz) to $1.5 \cdot 10^{-2}$ (Lake Zürich). Lake Baldegg, Lake Brienz and Lake Greifen were also outliers (>3rd quartile + 1.5 × inner quartile range). Abundance of *sul1* in the remaining lakes was between $1.1 \cdot 10^{-2}$ (Lake Neuchatel) and 1.5 10^{-3} (Lake Rot). Apart from a few exceptions (Lake Geneva and Lake Cauma), *sul2* was less prevalent than *sul1* in the sampled lakes (2–167 times), reaching maximum values of $3.4 \cdot 10^{-3}$ (Lake Sempach). High *sul2* gene abundance was found in lakes Geneva, Soppen, Cauma, and Melch, but none of the lakes fulfilled the outlier criterion. Abundance of *sul1* and *sul2* was not correlated ($R = 0.04$, $p > 0.8$).

Tetracycline resistance genes were much less abundant compared to sulfonamide resistance genes, in the investigated lakes (Fig. 2(B)) and for most of the lakes remained below the limit of detection (see Table A.1). tet(W), which was the most abundant of the three *tet* genes investigated, was detected and quantifiable in the lakes Zürich, Baldegg, Greifen and Melch. Similarly, tet(M) was quantified from the latter three lakes and additionally from Lake Soppen. Two more lakes (Lake Cauma and Türler) were tet(M)-positive but remained below the limit of quantitation. tet(B) was only detected in Lake Baldegg.

qnrA was not detected in the studied lakes. Due to these findings, *tet* genes and *qnrA* were not considered in the following analysis of possible determinants for ARG abundance.

3.2. Impact of lake and catchment characteristics on abundance of ARG

Calculation of Pearsons' correlation coefficients for data on land-use patterns and general lake characteristics versus abundance of *sul1* indicated that no single factor exerted a strong control over this parameter (Table A.4). The abundance of *sul2* showed the highest correlation coefficients with lake water retention time (RT, R-value: 0.61, $p < 0.05$) and a negative relation to urban areas (UA, R-value −0.52, $p < 0.05$).

Although none of the sampled lakes can be considered entirely pristine, a number of the sampled lakes ($n = 10$) had neither hospitals nor WWTPs in their catchments (see Table 2). These lakes were therefore a priori classified as "low impact" lakes. Comparing this group against the other lakes ("impacted", $n = 11$) showed no significant difference of the mean values for *sul1* and *sul2* gene abundance in the "impacted" group (mean ± standard deviation from the mean (SD): $sul1 = 2.97 \cdot 10^{-2} \pm 6.22 \cdot 10^{-2}$, mean $sul2 = 1.09 \cdot 10^{-3} \pm 1.14 \cdot 10^{-3}$) versus the "low impact" group ($sul1 = 4.97 \cdot 10^{-3} \pm 4.1 \cdot 10^{-3}$; $sul2 = 1.12 \cdot 10^{-3} \pm 1.11 \cdot 10^{-3}$; pairwise *t*-test, $p = 0.2$ and $p = 0.9$, respectively).

In order to test if combinations of lake and land use variables could better explain the abundance of *sul1* and *sul2* in the investigated lakes than single parameters, stepwise linear multiple regression was performed. Based on initial models [A1,2], stepwise variable elimination found [A1*] and [A2*] to represent the best models for *sul1* and *sul2*, respectively (Table 3). When considering land use variables normalized to either catchment size (CA, initial models [B1,2]) or lake volume (LV, initial models [C1,2]) instead, the best fits for explaining abundance of *sul1* are given in [B1*] and [C1*], respectively and of *sul2* in models [B2*] and [C2*], respectively (Table 3).

According to this analysis, abundance of *sul1* is best explained by the presence of WWTPs (CW) and hospitals (CH) in the lake catchments. Note that coefficients of the estimates (Table 3) show a negative relationship between *sul1* abundance in lakes and capacity of hospitals. Model selection for *sul1* was not affected by normalizing variables for land use to catchment area (CA, [B1*]) or lake volume (LV, [C1*]), confirming instead the positive relationship of *sul1* with the presence of WWTPs and its negative relation to hospitals in the lake catchments.

Abundance of *sul2* appears strongly related to long water retention times of lakes (RT) as well as high total phosphorous (TP) content (model [A2*]). Together with these variables *sul2*-abundance is also negatively related to the proportion (%) of urban areas (UA) in the catchment (see coefficients, Table 3), though this relation is not significant (see p-values, Table 3). As for *sul1*, these models for *sul2* abundance could be confirmed when normalizing variables for land use to CA [B2*] or LV [C2*]. In addition, when land use variables were normalized to CA, a positive relation of *sul2* to hospital capacity was revealed by model [B2*], but as for UA this relation was not significant (see p-values in Table 3).

3.3. Relationship between ARG abundance and microbial community structure in sampled lakes

Principal coordinates analysis of ARISA indicated that the majority of lakes contained a distinct microbial community at the time of sampling (Fig. 3). However, Lakes Burgäschi, Greifen, Zug, Baldegg, and Rumen

[1] ARG — either response variable *sul*1 or *sul*2.

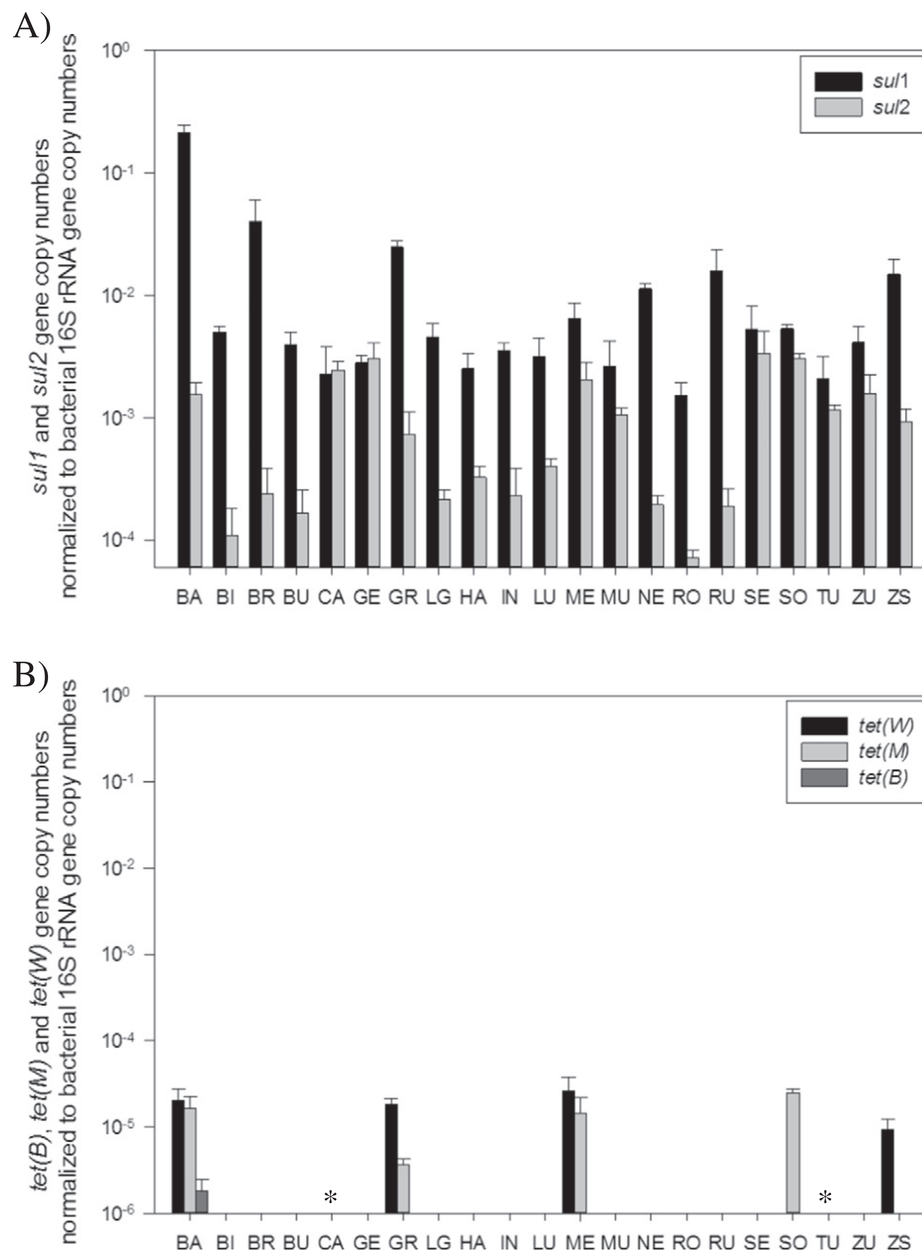

Fig. 2. Relative abundance of sulfonamide resistance genes (A) and tetracycline resistance genes (B) in 21 Swiss lakes. Error bars indicate standard deviations of extraction triplicates. An asterisk indicates lakes considered positive for tet(M), but below the limit of quantitation (compare Table A.1). 2-Column-fitting image.

had a very similar community composition (Fig. 3). The goodness of fit was 0.43.

Log-transformed centroid scores for each lake for the first 5 dimensions of ordinated ARISA data were used as explanatory variables in stepwise linear regression models with response variables *sul1* and *sul2*, (both arcsin-transformed), respectively. None of the models identified a significant relationship between any of the 5 dimensions and *sul1*. Only a marginally significant ($p < 0.1$) correlation between dimension 5 and *sul2* was observed (Adjusted $R^2 = 0.1$, $p = 0.08$). These results indicated that the abundance of the *sul* genes was not related to any major trends in microbial community composition.

4. Discussion

4.1. Impact of land use pattern on abundance of sul genes in freshwater lakes

One major goal of this study was to evaluate the impact of land use patterns in lake catchments on the abundance of ARGs. One of our assumptions was that agricultural, rather than urban impacts are the main drivers of elevated ARG abundance in lakes. Moreover, we assumed that an elevated inorganic nutrient content (total phosphorous, nitrate) in lakes, which is as an indicator for nutrient inputs from both human wastewaters and agriculture (fertilizers), may be related to prevailing resistance gene levels.

Lakes Baldegg and Greifen which exhibited both elevated levels of *sul1* and presence of *tet* genes, are in line with our expectations: both lakes are eutrophic and their catchment is mainly affected by agricultural activities (72%). Additionally, in the case of Lake Greifen, several important human contamination sources (hospitals and WWTPs) are present. In contrast, Lake Brienz which is an extremely oligotrophic lake and Lake Melch, an alpine mesotrophic lake, both have catchments dominated by natural environments (e.g., forests, data not shown). Yet, we measured elevated levels of *sul1* in Lake Brienz (outlier above upper quartile) and Lake Melch contained both *sul* and *tet* genes. In spite of the low proportions of agricultural and urban areas in the catchments of these lakes, several hospitals and WWTPs are present in the catchment

Table 3
Best-fitted models and coefficients selected for *sul1* and *sul2* by multiple linear regression: [A1,2*] using transformed data without normalization of land use variables to catchment area or lake volume, [B1,2*] transformed data with land use variables normalized to catchment area, or [C1,2*] lake volume. Abbreviations of variables are explained in Tables 1 & 2 of the manuscript.

Model	Estimate	Std. Error	t value	Pr (>|t|)	Multiple R-sq.	Adjusted R-sq.	p-Value
[A1*] sul1 ~ CW + CH					0.30	0.22	0.04*
CW	0.01255	0.0046	2.750	0.01*			
CH	−0.01030	0.0043	−2.386	0.03*			
[B1*] sul1 ~ CW/CA + CH/CA					0.31	0.24	0.03*
CW/CA	0.01535	0.0054	2.866	0.01*			
CH/CA	−0.0117	0.0051	−2.294	0.03*			
[C1*] sul1 ~ CW/LV + CH/LV					0.33	0.26	0.03*
CW/LV	0.00769	0.0026	3.002	0.01**			
CH/LV	−0.00619	0.0025	−2.454	0.02*			
[A2*] *sul2* ~ UA + TP + RT					0.64	0.54	0.01**
UA	−0.02477	0.0195	−1.273	0.23			
TP	0.00679	0.0026	2.575	0.03*			
RT	0.00679	0.0025	2.717	0.02*			
[B2*] *sul2* ~ CH/CA + UA + TP + RT					0.69	0.57	0.01**
CH/CA	0.00074	0.0005	1.35	0.21			
UA	−0.02454	0.01877	−1.307	0.22			
TP	0.00903	0.0030	2.973	0.01*			
RT	0.00679	0.0024	2.817	0.02*			
[C2*] *sul2* ~ UA + TP + RT					0.64	0.54	0.01**
UA	−0.02477	0.0195	−1.273	0.23			
TP	0.00679	0.0026	2.575	0.03*			
RT	0.00679	0.0025	2.717	0.02*			

Signif. codes: '***' <0.001, '**' <0.01, '*' <0.05.

of Lake Brienz and the number of cattle and pigs related to the lake volume of Lake Melch is comparatively high (73 and 75 animals, respectively, per 10^6 m^3). Hence, considering the trophic status of lakes and land use data of their catchment alone does not seem sufficient to obtain good estimates for human impact on lakes. Likewise, no significant difference between lakes with and without hospitals and WWTPs in their catchment could be found neither for *sul1* nor *sul2* and Pearsons' correlation analysis could not reveal any significant relation between WWTPs and these ARG. It is known, that in lakes with long lake water residence times (a year or more) pollutants may also be stored for extended periods (Wedepohl et al., 1990). In-line with this fact, the only positive relation that could be derived from correlation analysis of single parameters in this study was found for *sul2* abundance and lake water residence time. Nevertheless, taken together, these results suggest that abundance of ARGs in the sampled lakes is likely driven by complex interplays of different environmental and anthropogenic variables, rather than being related to any single factor.

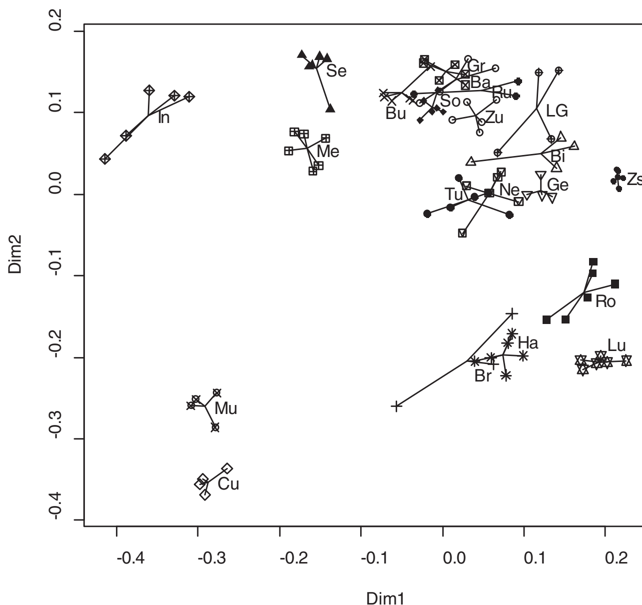

Fig. 3. Principle coordinates analysis of ARISA community profiles, showing community similarity in the first two dimensions. Symbols show extraction sample and technical replicates analyzed for each lake, and are connected by a line to the centroid for each lake. 1-Column-fitting image.

For this reason we carried out stepwise multiple linear regressions, considering all available environmental and catchment related variables. Interestingly, the only explanatory variables related positively to abundance of *sul1* identified by this approach were the presence and size of WWTPs in the catchment. Though hospitals were also identified as important parameters, their negative relationship to *sul1* abundance may indicate that indeed communal sewage alone may contribute to *sul1* pollution of lakes. The moderate R^2 of these linear models indicates that there must be other important drivers or stochastic effects, but the model was significant. This finding is also in line with observations in Lake Geneva (Czekalski et al., 2012) or Cache la Poudre river (Pruden et al., 2006) where inputs from WWTPs were found to increase *sul* gene abundance in natural waters. We can at present only speculate with regard to the negative impact of hospitals indicated by the model. The result is counter-intuitive as hospital wastewater is highly contaminated with resistant bacteria and ARGs (Baquero et al., 2008; Czekalski et al., 2012; Kümmerer, 2004), although the contribution to the total wastewater volume will usually be small. It would have to be determined whether the relationship is accidental or whether there are real indirect effects — e.g., absence of hospitals in the catchment may go along with a higher incidence of outpatient treatment with antibiotics, or could be correlated with less efficient sewage treatment in more rural areas.

As high abundance of *sul1* was not accompanied by high abundance of *sul2*, it is not surprising that selected models for *sul2* were composed of different explanatory variables. However, the lack of correlation between the abundance of these genes is in contrast to our previous studies (Czekalski et al., 2012, 2014) that showed a high degree of correlation between resistance genes along contamination gradients. The lack of correlation may thus indicate a lack of strong contamination sources, or heterogeneity within the contamination sources.

As for simple correlation analysis, stepwise linear regression likewise revealed a significant relation between *sul2* abundance and lake retention times in addition to total phosphorous content. The latter finding is in line with our hypothesis, that elevated nutrient contents in lakes, as a proxy for human impact, are related to elevated ARG content. Interestingly, when normalizing land use-variables to the size of catchment area (CA), a positive relation to hospital capacity (CH) in the catchment area was revealed for *sul2* (model [B2*]). Although variable CH/CA was not found to be individually significant ($p = 0.21$), this finding might provide some support for the idea that hospitals as well as WWTPs are important contamination sources of ARG, despite the negative correlation we found for hospitals and *sul1*. As the presence of hospitals and WWTPs can also be understood as proxies for urban activities, the antiproportional relationship between *sul2* abundance and the proportion of urban areas surrounding our studied lakes is somewhat contradictory. Urban areas can be composed of continuous and discontinuous building, indicating that the value of this variable may be quite heterogeneous and have less explanatory power. More in line with our finding of *sul2*-abundance being related to human-derived sources is the study of Pruden et al. (2006) at the Cache la Poudre river. They suggested *sul2* to be a sensitive indicator for urban inputs as they did not detect this gene at sites solely affected by agricultural activities. However, source tracking of both *sul* genes using antibiotic-resistance-gene molecular signatures revealed those genes to be indicators of both animal feeding operations and human sewage (Storteboom et al., 2010). *sul* genes were, therefore, considered as general indicators of pollution-impacted environments as they were low or absent in pristine environments.

The relatively high proportion of unexplained variance in our models indicates that *sul* abundances in the sampled lakes cannot be

entirely explained from our dataset. So far our hypothesis that agricultural settings have a stronger impact over direct human inputs could not be confirmed. Rather, our analysis would indicate wastewater as the most identifiable driver for *sul1* abundance. In contrast *sul2* abundance was related to an elevated trophic state in the lakes, which may be linked to both human wastewater as well as nutrient inputs from agriculture. Moreover, we found that the composition of the microbial communities does not seem to contribute to explaining *sul* gene abundance. We conclude that *sul* gene abundance is unlikely to be tied to the abundance of a few major species in the bacterioplankton, which would confuse our interpretation. This result also indicates that typical drivers of bacterial community composition of freshwater bacteria, e.g., pH, nutrient availability, amount and quality of organic carbon, and predation (Köllner et al., 2013; Newton et al., 2011) are likewise unlikely to be major drivers of *sul* gene abundance.

Finally, lake bacterial communities are known to be strongly determined by temporal trends (Yannarell et al., 2003). While all lakes were sampled in summer, temporal variability may have contributed to the poor explanatory power of our dataset. This aspect will have to be studied in more detail on a smaller number of lakes.

4.2. Background and elevated levels of ARG in sampled Swiss lakes

The abundance of sulfonamide resistance genes in the sampled freshwater lakes may indicate both: pollution with ARG from the catchment as well as a greater potential of these genes to establish in natural microbial populations in the water sheds as compared to the investigated tetracycline resistance genes. Similar observations on the abundance of these two classes of ARG have been made when investigating their fate after sewage discharge in the Vidy bay in Lake Geneva (Czekalski et al., 2012, 2014) and along the Cache la Poudre River in northern Colorado which is affected by different land use pattern (Pei et al., 2006; Pruden et al., 2006; Storteboom et al., 2010). Ratios of both *sul* genes normalized to eubacterial 16S rRNA genes measured in sediments of the Cache la Poudre river in Northern Colorado ranged from a low level of 10^{-6} at a pristine upstream site up to 10^{-3} at a site heavily polluted due to both urban and agricultural activities. Interestingly, these ratios are generally lower than those measured in the contaminated sediments in Vidy bay (Czekalski et al., 2012) and in the water column of the sampled lakes (this study), where ratios of $\sim 10^{-3}$ would be assumed to represent background levels of *sul1*. The abundance of both *sul* genes varied considerably among the sampled lakes — the highest abundance being approximately 140 and 50 times higher than the lowest measured abundance, for *sul1* and *sul2*, respectively. Taking the 75% percentile as guidance, abundances below $1.5 \cdot 10^{-2}$ and $3.4 \cdot 10^{-3}$ of *sul1* and *sul2*, respectively, seem to betypical for Swiss lakes. The low impact group of lakes, with *sul1* and *sul2* gene abundance ranging between $1.5 \cdot 10^{-3}$ to $1.6 \cdot 10^{-2}$ and $3.1 \cdot 10^{-3}$–$7.2 \cdot 10^{-5}$, respectively, provides a useful baseline against which contamination with *sul* genes can be evaluated. Based on these results, *sul* abundances we measured in our previous work in Lake Geneva water near a pump for drinking water preparation (mean $7.3 \cdot 10^{-1} \pm$ (SD)$9 \cdot 10^{-2}$ and mean $4 \cdot 10^{-2} \pm$ (SD) $3 \cdot 10^{-2}$ for *sul1* and *sul2*, respectively, (Czekalski et al., 2012)) would appear to be above the baseline range, which might suggest a potential influence of the WWTP effluent discharged into the lake nearby. Such an impact has also been indicated by sediment data (Czekalski et al., 2014), whereas the lake at large ($2.8 \cdot 10^{-3} \pm$ (SD) $3.9 \cdot 10^{-4}$ and $3.1 \cdot 10^{-3} \pm$ (SD) $3.1 \cdot 10^{-3}$ measured at the center of the lake for *sul1* and *sul2*, respectively) is not subject to ARG pollution.

While the above discussion demonstrates the value of a reference dataset, more work remains to be done: our ARG data-set is composed of only a few sampling campaigns, standardized protocols for evaluating ARG pollution are currently lacking, and very little is known about the seasonal variability of ARG abundance in lakes. Therefore, more thorough investigations would be necessary to establish reliable thresholds for diagnosing ARG pollution.

Tetracycline resistance genes were mostly below detection in our assays. Hence, their presence in quantifiable amounts is by itself interpreted as an increased level in excess of background resistance, e.g., due to elevated pollution from the catchment. Unfortunately it is beyond the scope of our study to identify the exact sources of ARG measured in the sampled lakes and available tools and methods to do so are scarcely available. For instance, Storteboom et al. (2010) have determined antibiotic-resistance-gene molecular signatures for various *tet* genes (not however the ones detected in our study) and *sul1* and *sul2*. Whereas specific *tet* genes could be ascribed to either agricultural or human waste inputs, *sul* genes originated from any of the considered contamination sources but were only scarcely detected at pristine sites. This seems coherent, given the fully synthetic origin of sulfonamide antibiotics. Thus, it must be assumed that the widespread abundance of *sul* genes in Swiss lakes rather results from a long history of anthropogenic pollution, and does not represent a generally high natural (preceding the antibiotics era) abundance of these genes. Long term trends of increasing resistance gene abundance have been demonstrated e.g., in soil (Knapp et al., 2009). However, additional studies on aquatic environments e.g., on lake sediments as historical archives would be needed to confirm this hypothesis. The long-term usage of sulfa-drugs, despite their reduced utility in human medicine today (Sköld, 2000), thus may give an insight into how the resistance situation in the environment may evolve in the case of other, currently still more valuable (fully synthetic) antibiotics, such as fluoroquinolones.

It is known that the origin of *qnr*A is a natural bacterium, *Shewanella algae*, prevalent in freshwater and marine environments (Poirel et al., 2005). *qnrA* was also detected recently in *Aeromonas* species isolated from Lake Lugano in Switzerland (Picao et al., 2008), which however was not included in our survey. Having this information in mind, we conclude that not detecting *qnrA* using qPCR directly on environmental DNA-extracts does not necessarily mean it is absent in the sampled lakes. It is more likely that its abundance is not sufficient in order to be tracked by the currently available sensitivity of the applied qPCR methods, or that primer specificity is too narrow for capturing the diversity of *qnrA* present in environmental reservoirs. It also seems recommendable to accompany qPCR based screenings with enrichment steps for potential bacterial carriers of *qnrA* for obtaining more comprehensible results, as suggested previously (Czekalski et al., 2012). We conclude that natural *qnrA* levels in freshwater lakes are below detection given the current sensitivity of the applied molecular tools, so that detecting them in quantifiable amounts would indicate strong contamination, e.g., by sewage (Cummings et al., 2010).

4.3. Alternative explanations for resistance gene abundances

The abundance of resistance genes measured in this study, which were determined in near-surface water samples taken away from any local contamination sources, may reflect the establishment of resistance genes in the natural bacterioplankton communities rather than a contemporary impact of the discharge of resistant human and animal derived fecal or wastewater bacteria. Thus the observed patterns may reflect historical, evolutionary, or environmental conditions that favored the establishment of resistance genes in parts of the environmental bacterial community. These factors may not be well-represented in our explanatory dataset. Future work could therefore include a more thorough analysis of the historical development or investigations of the environmental parameters that may determine horizontal gene transfer and positive selection for resistance genes (e.g., concentrations of antibiotics). Including the analysis of sediment samples in future similar surveys may also provide further insights.

4.4. Conclusions

In spite of the remaining insecurity regarding the main drivers of antibiotic resistance gene abundance in lakes, our study highlights the potential of some ARG genes to prevail in freshwater environments and likewise the potential of freshwater lakes to be important reservoirs for maintaining and potentially disseminating ARG, as discussed previously (Jones et al., 1986; Young, 1993). Whereas a number of previous studies revealed ARG pollution in close proximity of contamination sources (Cummings et al., 2010; Czekalski et al., 2012; Graham et al., 2010; West et al., 2010), the outcome of our study indicates that, at least with respect to *sul1* genes, even background resistance of freshwater lakes may be impacted by anthropogenic activities in the catchment areas, such as wastewater discharge. Thus our findings support the recommendation by Baquero et al. (2008), that preventing mixing of human and animal-derived microbiota with environmental bacteria, e.g., by specific waste treatment, seems advisable. Such measures may help to restrict the risk of ARG accumulating in the aquatic environment which humans are exposed to via the food chain or drinking water and could thus help to limit resistance gene transfer to pathogens. This study also provides a first baseline for comparison of ARG abundance in freshwater systems that will be valuable in distinguishing contamination from the resistance background in future studies.

In order to improve conclusions on the impact of land use patterns on the abundance of resistance genes in the studied lakes, both the explanatory dataset and the underlying cause–effect model could be improved in many aspects in future work. Incorporating data on antibiotic consumption in the considered human and agricultural settings would be of great value but are unfortunately not available at the required spatial resolution at the moment. Also, concentrations of antibiotics and other biocides in the lakes (and tributaries) would be valuable parameters to check. Spatial weighing of sources and transport aspects could be incorporated. Moreover, temporal effects, e.g., rain fall events and seasonal variations of ARG ratios should be considered.

Acknowledgments

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Appendix A

Table A.1
Ct-values (and gene copies-c#-per 5 μl DNA-extract) for limit of quantitation (LOQ) and limit of detection (LOD) for qPCR assays. n.d.: not determined due to Ct-values > 40 cycles.

Gene	LOQ (c#/5 $^{-1}$μl)	LOD
sul1	33 (300)	n.d.
sul2	35 (300)	n.d.
tet(B)	34 (50)	35.1
tet(M)	34.7 (35)	37.1
tet(W)	30.6 (100)	32.4
qnrA	31.4 (30)	n.d.
16S	34 (170)	36

Table A.2
Akaike Information Criterion (AIC) values, AIC differences (Δi), likelihood of each model ((gi x)), and Akaike weights (w_i) for tested model sets with *sul1* abundance as the response variable. A) data without normalization of land use variables to catchment area or lake volume, B) data with land use variables normalized to catchment size and C) data with land use variables normalized to lake volume. Abbreviations are explained in Tables 1 & 2 of the manuscript.

[Model]	R-code	AIC	Δi	$\mathcal{L}$(gi x)	w_i	Evidence ratio
A)						
[A1*]	sul1 ~ CW + CH	−38.9786	0	1	0.67	1
	sul1 ~ CW + CH + NP	−37.5494	1.429	0.49	0.33	2.04
	sul1 ~ CW + CH + NP + NO	−24.0106	14.968	5.6E−04	3.8E−04	1.78E + 03
	sul1 ~ CW + CH + NP + AA + NO	−22.2206	16.758	2.3E−04	1.5E−04	4.35E + 03
	sul1 ~ CW + CH + NP + AA + NO + RT	−15.3357	23.643	7.3E−06	4.9E−06	1.36E + 05
	sul1 ~ CW + CH + NP+ UA + AA + NO + RT	−13.8486	25.130	3.5E−06	2.3E−06	2.86E + 05
[A1]	sul1 ~ CW + CH + NP + UA + AA + TP + NO + RT	−12.0725	26.906	1.4E−06	9.6E−07	6.96E + 05
			$\sum\mathcal{L}$(gi x)	1.4902		
B)						
[B1*]	sul1 ~ CW/CA + CH/CA	−39.5093	0	1	0.70	1
	sul1 ~ CW/CA + CH/CA + TP	−37.8516	1.65773	0.44	0.30	2.29
	sul1 ~ CW/CA + CH/CA + TP + NO	−23.8439	15.6655	4.0E−04	2.8E−04	2.52E + 03
	sul1 ~ CW/CA + CH/CA + AA + TP + NO	−22.1986	17.3107	1.7E−04	1.2E−04	5.74E + 03
	sul1 ~ CW/CA + CH/CA + AA + TP + NO + RT	−14.4847	25.0247	3.7E−06	2.6E−06	2.72E + 05
	sul1 ~ CW/CA + CH/CA + UA + AA + TP + NO + RT	−12.4869	27.0224	1.4E−06	9.4E−07	7.38E + 05
[B1]	sul1 ~ CW/CA + CH/CA + NP/CA + UA + AA + TP + NO + RT	−10.4882	29.021	5.0E−07	3.5E−07	2.00E + 06
			$\sum\mathcal{L}$(gi x)	1.43712		
C)						
[C1*]	sul1 ~ CW/LV + CH/LV	−40.1382	0	1	0.68	1
	sul1 ~ CW/LV + CH/LV + NP/LV	−38.6435	1.49461	0.47	0.32	2.11
	sul1 ~ CW/LV + CH/LV + NP/LV + NO	−24.6060	15.5322	0.00	0.00	2.36E + 03
	sul1 ~ CW/LV + CH/LV + NP/LV + NO + TP	−23.2567	16.8814	0.00	0.00	4.63E + 03
	sul1 ~ CW/LV + CH/LV + NP/LV + UA + TP + NO	−21.2698	18.8684	0.00	0.00	1.25E + 04
	sul1 ~ CW/LV + CH/LV + NP/LV + UA + TP + NO + RT	−13.3643	26.7739	0.00	0.00	6.51E + 05
[C1]	sul1 ~ CW/LV + CH/LV + NP/LV + UA + AA + TP + NO + RT	−11.3682	28.7699	0.00	0.00	1.77E + 06
			$\sum\mathcal{L}$(gi x)	1.47436		

Table A.3
Akaike Information Criterion (AIC) values, AIC differences (Δi), likelihood of each model ((gi x)), and Akaike weights (w_i) for tested model sets with *sul2* abundance as the response variable. A) data without normalization of land use variables to catchment area or lake volume, B) data with land use variables normalized to catchment size and C) data with land use variables normalized to lake volume. Abbreviations are explained in Tables 1 & 2 of the manuscript.

[Model]	R-code	AIC	Δi	$\mathcal{L}$(gi x)	w_i	Evidence ratio
A)						
[A2*]	*sul2* ~ UA + TP + RT	−86.6696	0.00	1.00	0.24	1.00
	sul2 ~ TP + RT	−86.6087	0.06	0.97	0.23	1.03
	sul2 ~ CH + UA + AA + TP + NO + RT	−86.0087	0.66	0.72	0.17	1.39
	sul2 ~ CH + UA + TP + RT	−86.5713	0.10	0.95	0.23	1.05
	sul2 ~ CH + UA + NO + TP + RT	−85.3069	1.36	0.51	0.12	1.98
	sul2 ~ CW + CH + UA + AA + TP + NO + RT	−84.2151	2.45	0.29	0.07	3.41
[A2]	*sul2* ~ CW + CH + NP + UA + AA + TP + NO + RT	−82.6758	3.99	0.14	0.03	7.37
			$\sum\mathcal{L}$(gi x)	4.15		
B)						
[B2*]	*sul2* ~ CH/CA + UA + TP + RT	−87.1805	0.00	1.00	0.23	1.00
	sul2 ~ CH/CA + TP + RT	−86.8136	0.37	0.83	0.19	1.20
	sul2 ~ TP + RT	−86.6087	0.57	0.75	0.17	1.33
	sul2 ~ CH/CA + UA + AA + TP + NO + RT	−86.5918	0.59	0.75	0.17	1.34
	sul2 ~ CH/CA + UA + TP + NO + RT	−86.1435	1.04	0.60	0.13	1.68
	sul2 ~ CH/CA + NC/CA + UA + AA + TP + NO + RT	−85.2147	1.97	0.37	0.08	2.67
[B2]	*sul2* ~ CW/CA + CH/CA + NP/CA + UA + AA + TP + NO + RT	−83.2413	3.94	0.14	0.03	7.17
			$\sum\mathcal{L}$(gi x)	4.43796		
C)						
[C2*]	*sul2* ~ UA + TP + RT	−86.6696	0	1	0.21	1.00
	sul2 ~ CH/LV + UA + TP + RT	−86.6474	0.02221	0.98896	0.21	1.01
	sul2 ~ TP + RT	−86.6087	0.06099	0.96997	0.21	1.03
	sul2 ~ CH/LV + UA + AA + TP + NO + RT	−85.8373	0.83235	0.65956	0.14	1.52
	sul2 ~ CH/LV + UA + TP + NO + RT	−85.6748	0.9948	0.60811	0.13	1.64
	sul2 ~ CW/LV + CH/LV + UA + AA + TP + NO + RT	−84.4699	2.19972	0.33292	0.07	3.00
[C2]	*sul2* ~ CW/LV + CH/LV + NP/LV + UA + AA + TP + NO + RT	−82.5167	4.15292	0.12537	0.03	7.98
			$\sum\mathcal{L}$(gi x)	4.68489		

Table A.4
Pearsons correlation coefficients (and p-values) calculated for variables characterizing sampled lakes and activities in their catchment area. Bold values indicate significant correlation (R > 0.5). Variables of lake data (LV, TP, NO, RT, Table 1) and land use-data (CA, NW, CW, NH, CH, NC, NP, Table 2) were log-transformed and proportional (%, UA & AA, Table 2) variables (including *sul1* and *sul2*) were arcsin-transformed prior to analysis (left column). In addition, land use variables were normalized to either catchment area (middle column) or volume of lakes (right column) and hereafter log transformed prior to analysis. Abbreviations of variables are explained in Tables 1 & 2 of the manuscript.

Data	Transformed				Normalized to catchment area				Normalized to lake volume			
	sul1		*sul2*		*sul1*		*sul2*		*sul1*		*sul2*	
LV	0.09	(>0.5)	0.12	(>0.1)								
TP	−0.09	(>0.5)	0.11	(>0.1)								
NO	0.27	(>0.3)	0.01	(>0.1)								
RT	0.06	(>0.5)	**0.61**	**(0.01)**								
CA	0.10	(>0.5)	−0.03	(>0.5)								
NW	0.17	(>0.5)	−0.07	(>0.1)	0.29	(0.21)	−0.01	(>0.5)	0.32	(0.16)	−0.03	(>0.5)
CW	0.27	(>0.5)	−0.01	(>0.1)	0.33	(0.14)	0.02	(>0.5)	0.33	(0.14)	−0.01	(>0.5)
NH	−0.02	(>0.5)	−0.03	(>0.1)	0.04	(>0.5)	0.10	(>0.5)	0.00	(>0.5)	−0.01	(>0.5)
CH	0.00	(>0.5)	0.00	(>0.1)	0.01	(>0.5)	−0.01	(>0.5)	0.00	(>0.5)	0.00	(>0.5)
NC	0.07	(>0.5)	0.08	(>0.1)	−0.01	(>0.5)	0.18	(0.43)	0.00	(>0.5)	0.13	(>0.5)
NP	0.13	(>0.5)	0.10	(>0.1)	0.08	(>0.5)	0.19	(0.4)	0.04	(>0.5)	0.23	(0.30)
NC + NP	0.09	(>0.5)	0.10	(>0.1)	0.02	(>0.5)	0.21	(0.37)	−0.05	(>0.5)	0.15	(>0.5)
UA	−0.04	(>0.5)	**−0.52**	**(0.02)**								
AA	0.16	(>0.5)	0.06	(>0.1)								
sul1	1		0.04	(>0.1)								
sul2			1									

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