

ORIGINAL ARTICLE

Wastewater as a point source of antibiotic-resistance genes in the sediment of a freshwater lake

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Antibiotic-resistance genes (ARGs) are currently discussed as emerging environmental contaminants. Hospital and municipal sewage are important sources of ARGs for the receiving freshwater bodies. We investigated the spatial distribution of different ARGs (*su1*, *su2*, *tet(B)*, *tet(M)*, *tet(W)* and *qnrA*) in freshwater lake sediments in the vicinity of a point source of treated wastewater. ARG contamination of Vidy Bay, Lake Geneva, Switzerland was quantified using real-time PCR and compared with total mercury (THg), a frequently particle-bound inorganic contaminant with known natural background levels. Two-dimensional mapping of the investigated contaminants in lake sediments with geostatistical tools revealed total and relative abundance of ARGs in close proximity of the sewage discharge point were up to 200-fold above levels measured at a remote reference site (center of the lake) and decreased exponentially with distance. Similar trends were observed in the spatial distribution of different ARGs, whereas distributions of ARGs and THg were only moderately correlated, indicating differences in the transport and fate of these pollutants or additional sources of ARG contamination. The spatial pattern of ARG contamination and supporting data suggest that deposition of particle-associated wastewater bacteria rather than co-selection by, for example, heavy metals was the main cause of sediment ARG contamination.

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Introduction

Since their introduction into medical practice in the 1930s, antibiotics have saved millions of lives, but the increasing numbers of bacterial pathogens that are becoming resistant to antibiotics is a growing cause of concern. Research initially focused on clinical settings as the main site of the spread and evolution of antibiotic resistance. Over the course of the past two decades, however, researchers have increasingly broadened their focus to include the environment as a source of resistance genes and as a site of antibiotic-resistance evolution (Kümmerer, 2004). The existence of a natural environmental resistance gene pool, which includes all known clinical resistance mechanisms, has been discovered (D'Costa *et al.*, 2006; Aminov, 2009). Consequently, bacterial pathogens are assumed to acquire

resistance traits from the environment via horizontal gene transfer (Aminov, 2009; Martinez, 2009a). Moreover, various studies have demonstrated that wastewater and animal waste contain large numbers of resistant bacteria, which can pass through the wastewater treatment plant (WWTP) and reach the receiving water bodies (Teuber, 2001; Kümmerer, 2004; Jury *et al.*, 2011; Rizzo *et al.*, 2013; Zurfluh *et al.*, 2013). There is growing concern that continuous discharge of these contaminants may, at least locally, lead to an increase in the natural resistance background levels and thus enhance the likelihood of antibiotic-resistance genes (ARGs) being transferred back to human and animal commensals or even pathogens (Kim and Aga, 2007; Martinez, 2009b; Cantas *et al.*, 2013). In the long term, this 'environmental loop' could contribute to the spread of resistance in pathogens and undermine the effectiveness of current and future antibiotics.

Lake Geneva is the largest freshwater body in Western Europe and receives treated wastewaters from the surrounding cities. The largest WWTP is located in Lausanne and discharges treated wastewater and occasionally bypass water into Vidy Bay.

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The bay's sediments have been reported to be heavily polluted by fecal-indicator bacteria, heavy metals, nitrogen and phosphorus (Poté *et al.*, 2008). Micropollutants, including antibiotics (Bonvin *et al.*, 2011), as well as multiresistant bacteria and ARGs pass through the WWTP and are subsequently discharged in considerable amounts into Vidy Bay (Czekalski *et al.*, 2012). Low levels of multiresistant bacteria and ARGs were found even 3.2 km from the WWTP discharge pipe, where lake water is pumped for drinking water production (Czekalski *et al.*, 2012). However, it remained unclear whether this represented contamination or the (unknown) natural resistance background level of Lake Geneva.

Molecular tools, such as PCR and quantitative real-time PCR, have been developed for many ARGs (Heuer and Smalla, 2007; Walsh *et al.*, 2011). A limited number of studies have applied molecular methods to aquatic environments. Primarily, rivers impacted by different contamination sources have been considered (Pei *et al.*, 2006; Pruden *et al.*, 2006; Graham *et al.*, 2010). In these systems, the horizontal transport of contaminants is directional and constrained by the river channel and therefore relatively well defined. This is also true for most of the sampling points in the study of LaPara *et al.* (2011), which followed the input of ARGs with wastewater into the St Louis river and transport into Lake Superior. In freshwater lakes, complex transport processes prevail, dominated, for example, by temporally variable wind regimes, currents and stratification. Despite the high relevance of lakes as sources of drinking water, food and as a place for recreational activities, the dynamics of antibiotic-resistance contamination in lakes that are receiving waters for municipal wastewater has not been thoroughly investigated. It is currently unknown, for example, at which spatial scales a local source of antibiotic-resistant bacteria affects water and sediment in a receiving lake.

The present study aims to characterize the spatial impact of Lausanne's WWTP discharge on the concentration of several plasmid-mediated ARGs in the lake sediment. The tested ARGs confer resistance to different classes of broad-spectrum antibiotics (sulfonamides: *sul1*, *sul2*, tetracyclines: *tet(M)*, *tet(B)*, *tet(W)*) and fluoroquinolones: *qnrA*), which differ in their origin (for example, natural versus synthetic), time of introduction, resistance mechanisms and clinical relevance. Sulfonamides were the first synthetic antibacterials. Tetracyclines, isolated from *Streptomyces*, followed in 1948. Fluoroquinolones were introduced during the 1970s and are, of the three substance classes, currently the most frequently used in clinics. ARGs were compared with an already well-studied WWTP-released contaminant, total mercury (THg), for which background levels in Lake Geneva are known from previous studies (Arboille *et al.*, 1989; Poté *et al.*, 2008). Mapping of the two-dimensional spatial pattern of the investigated ARGs and THg

was carried out using a geographic information system (ArcGIS). Further data on the microbial community structure in wastewater and sediment samples and sediment properties were considered in order to interpret the observed spatial trends of ARGs in Vidy bay sediments.

Materials and methods

Study site description and sampling campaigns

For a detailed description of the Vidy Bay study site, please refer to Czekalski *et al.* (2012). Lausanne (population 214 000) is the biggest city in the Lake Geneva area. The city has no pharmaceutical industries but several health-care centers. The largest is the Centre Hospitalier Universitaire Vaudois, an important site of antibiotic consumption, which discharges $\sim 410 \text{ m}^3 \text{ day}^{-1}$ of raw wastewater into the municipal sewage system (Blanc, 2010). The conventional WWTP of Lausanne treats the bulk of the wastewater it receives with a chemical phosphate removal process and an aerobic biological treatment, followed by clarification. Sludge is recycled or incinerated. Only part of the water receives a more elaborate biological denitrification treatment (Supplementary Figure S1). Disinfection is not performed. Treated wastewater and occasionally untreated sewer overflow (bypass) is discharged at an average rate of $86.631 \text{ m}^3 \text{ day}^{-1}$ ($1\text{--}3 \text{ m}^3 \text{ s}^{-1}$ up to $5\text{--}6 \text{ m}^3 \text{ s}^{-1}$ at low and peak flow, respectively) (Vioget *et al.*, 2011). The effluent pipe discharges the treated water 750 m offshore at a depth of 35 m into the Vidy Bay of Lake Geneva (Figure 1, sampling point STEP ('station d'épuration')). The rivers Chamberonne and Flon (sampling points CHAM and FLON) form part of the city's combined sewer overflow system and also discharge into the bay ($1\text{--}3 \text{ m}^3 \text{ s}^{-1}$, Goldscheider *et al.*, 2007). The main form of agricultural land use in their catchment areas is vineyards. Intensive animal farming is not prevalent. About 3.2 km south-west from STEP, lake water is pumped for drinking water production (sampling point DWP).

Short sediment cores were taken at 22 sites during two sampling campaigns in August 2011 (Figure 1). Sediment cores were retrieved as described previously (Czekalski *et al.*, 2012). Sediment cores were transported to the laboratory within 8 h and stored at 4°C for a maximum of 48 h until processing. Three subsamples of 0.5 g (wet weight) from the sediment surface layer (0–3 cm) of each core were transferred to sterile 2-ml screw cap tubes. After shock-freezing in liquid nitrogen, the tubes were stored at -80°C until DNA extraction. For a subset of sites, additional subsamples of surface sediments were taken, immediately preserved by freezing at -20°C and freeze-dried to analyze THg, grain size and organic matter content.

Details on additional water samples taken in 2009 and 2010 from Lausanne's sewage system and lake

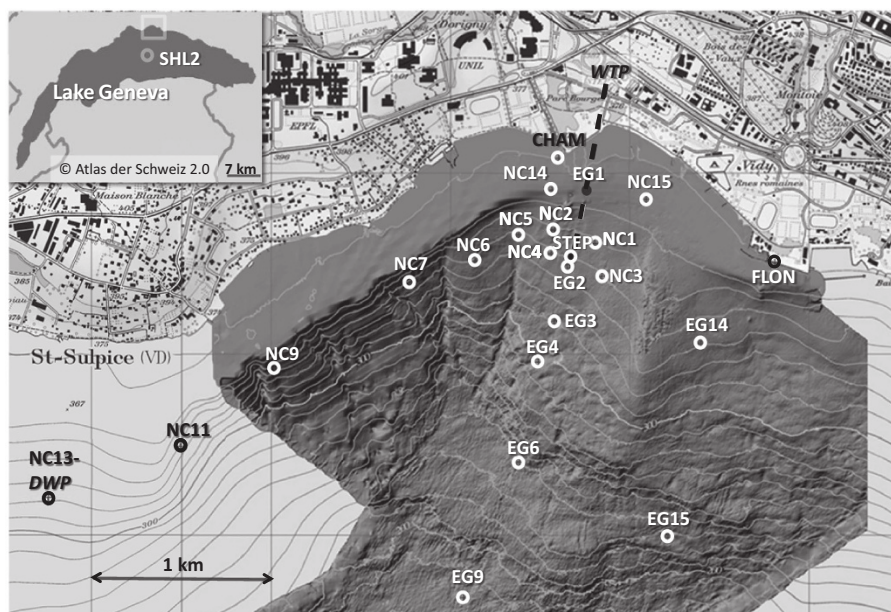


Figure 1 Map of Vidy Bay, indicating its location in Lake Geneva, 22 sites from which sediments were sampled, and the two river mouths of the Flon and Chamberonne (CHAM). The WWTP discharge pipe is indicated by a dashed line starting at Lausanne's WWTP to the point of discharge, the STEP site. Bathymetric data provided by Anh-Dao Le thi and Walter Wildi, Institut F.A. Forel. Maps used with permission of swisstopo (Art. 30 GeoIV): PK50©2007, 2005©swisstopo.

water in Vidy Bay that were analyzed by plate counts of resistant bacteria and qPCR assays are given in the Supplementary Information.

DNA extraction

DNA from the three subsamples from each sampling site was extracted, quantified and quality-controlled as described in Czekalski *et al.* (2012). Briefly, cells were lysed using bead-beating and freeze-thaw cycles, and DNA was purified using a phenol-chloroform extraction protocol. Extraction blanks omitting sediment were prepared to check for contamination sources. DNA quality was checked via agarose gel electrophoresis and ultraviolet-absorbance (Nanodrop, Thermo Fisher Scientific Inc., NanoDrop products, Wilmington, DE, USA) and quantified using the Quant-iT PicoGreen DNA quantification kit (Invitrogen, Basel, Switzerland). Extraction blanks yielded negative results. DNA pellets were resuspended in 50–100 μ l of Tris-EDTA buffer and stored at -20°C until further analysis.

qPCR assays performed with sediment, lake and wastewater DNA extracts

Six different ARGs (*sul1*, *sul2*, *tet(B)*, *tet(M)*, *tet(W)* and *qnrA*) and bacterial 16S rRNA gene fragments were quantified using the published protocols, primers and probes (Supplementary Table S1). Standard curves were prepared from serial 10-fold dilutions of plasmid DNA containing the respective target gene in a range of 3×10^6 to 30 gene copies. The preparation of control plasmids and standard curves for *sul1* and *sul2* was as described previously

(Heuer and Smalla, 2007; Heuer *et al.*, 2008). For *tet(B)*, *tet(M)*, *tet(W)* and *qnrA*, standard curves were prepared from cloned PCR products as described for the 16S rRNA gene in Czekalski *et al.* (2012). qPCR assays for *sul*, *tet* and 16S rRNA genes were performed in 96-well plates using TaqMan Environmental Master Mix 2.0 (Life Technologies Corp., Applied Biosystems, Carlsbad, CA, USA), which is optimized for samples with confirmed or expected presence of PCR inhibitors. *qnrA* was amplified using QuantiTect SYBR Green PCR chemistry (Qiagen, Hombrechtikon, Switzerland). In brief: each 25- μ l reaction mixture contained 1 \times of either Environmental or SYBR green Master Mix, primers, probe and MgCl_2 according to the published methods (Supplementary Table S1) and 5 μ l of template DNA or standard. All qPCR assays were performed in technical triplicates on each extract, standard and negative controls (no template control: salmon sperm DNA, 10 $\text{ng} \mu\text{l}^{-1}$ in order to control for specificity; PCR blanks: nuclease-free water; and extraction blanks: see above). qPCR reactions were performed on a 7500 Fast Real-Time PCR System (Applied Biosystems) and analyzed using default settings.

All negative controls resulted either in no amplification or a higher threshold cycle (Ct) than the most diluted quantification standard. A sample was considered to be below limit of detection (LOD) for a target gene if ≥ 2 out of 3 technical replicates were negative or if sample Ct values were \geq Ct of negative controls. Samples above LOD were considered to be below the limit of quantification when the s.d. of Ct values of methodological triplicates was >0.5 and their Ct value was higher than the Ct of the most

diluted standard whose standard deviation of Ct values was ≤ 0.5 .

For each PCR assay, the qPCR efficiency was calculated from the slope of the standard curve ($E = 10^{(-1/\text{slope})}$; Supplementary Table S2). For a subset of seven samples and for one target gene (*sul2*), the effect of inhibitors in the sample matrix on amplification in qPCR assays was evaluated directly. Each of the samples was spiked with 10^5 copies of quantification standard DNA and amplified together with the same set of non-spiked samples and control DNA. The presence of inhibitors was evaluated according to Pei *et al.* (2006) by calculating the suppression factor *S*. *S* varied between 1.1 and 1.4, indicating that inhibition was negligible (Supplementary Table S3).

Automated ribosomal intergenic spacer analysis (ARISA)-PCR and sequencing

In all, 1:10 dilutions of DNA extracts were subjected to ARISA using general bacterial primers (Supplementary Table S1) and PCR conditions according to Yannarell *et al.* (2003) with slight modifications (Bürgmann *et al.*, 2011). A total of $10 \text{ ng } \mu\text{l}^{-1}$ of *Escherichia coli* DNA served as a positive control, $10 \text{ ng } \mu\text{l}^{-1}$ of salmon sperm DNA and nuclease-free water served as no template control and PCR blank, respectively.

Samples were run on an ABI 3130xl capillary sequencer as described previously (Bürgmann *et al.*, 2011). Data analysis was performed using Gene Mapper Software version 4.0 (Applied Biosystems) considering only peaks with sizes between 350 and 1250 bp and a minimum peak height of 125 fluorescence units. ARISA data were subjected to the automatic and interactive binning scripts (Ramette, 2009) for R (version 2.14.2, (2012)) using a window size of 2.5 bp.

Hg analysis

THg concentrations were determined by cold vapor atomic absorption spectrophotometry after dry mineralization and pre-concentration of Hg by means of amalgamation on a gold trap (Szaková *et al.*, 2004), using an automatic mercury analyzer (Model AMA 254, Altec, Prague, Czech Republic) under the following conditions: typical sample mass 50–100 mg, drying time 45 s, decomposition time 150 s and waiting time (necessary for quantitative trapping of released mercury on the gold amalgamator) 45 s. All analyses were run in duplicates. The relative error was usually $\pm 5\%$ and always under $\pm 10\%$ (Roos-Barracough *et al.*, 2002). The limit of detection is 0.01 ng, and the working range is between 0.05 to 600 ng (Yang and Pan, 2007). Concentrations obtained for repeated analyses of certified reference materials never exceeded the published range of concentration ($0.091 \pm 0.008 \text{ } \mu\text{g g}^{-1}$ for MESS-3).

Sediment characterization and nutrient content

The sediment grain size was measured using a Coulter LS-100 laser diffractometer (Beckman Coulter, Fullerton, CA, USA), following 5-min ultrasonic dispersal in de-ionized water according to the method of Loizeau *et al.* (1994).

The sediment total organic matter content was estimated from loss on ignition at 550 °C for 1 h in a laboratory oven (LE14/11, Nabertherm, Lilienthal, Germany).

Data analysis, visualization and statistics

Total copy numbers of all target genes were normalized to sediment sample wet weight (g^{-1} wwt). This is referred to as ARG concentration from here on. Additionally, ARG copy numbers were normalized to 16S rRNA gene copy numbers and reported as percentages, as an indicator of the relative abundance of resistance genes within the bacterial population. The number copies of the 16S rRNA genes per cell varies among bacterial species and may thus vary between samples, but keeping this caveat in mind we refer to this measure as 'ARG abundance' from here on. Interpolation maps were created with ArcGIS (ESRI ArcMap 10, Redlands, CA, USA) with the Geostatistical Analyst extension, using the Inverse Distance Weighted interpolation algorithm with power functions from 1 to 10 and a smoothing factor of 0.2.

In order to evaluate similarity of the distribution patterns between resistance genes and between resistance genes and Hg concentrations, as well as between ARGs and distance from the contamination source, correlation and regression analysis were performed in MS Excel 2010 (Microsoft, Redmond, WA, USA), using the Data Analysis Tool.

Community similarity analysis was performed based on Hellinger-transformed ARISA peak area data in R version 2.14.2 (R Core Team, 2012), using the BiodiversityR and vegan packages (Oksanen *et al.*, 2012; Kindt and Coe, 2005). Principle coordinates analysis was performed using the Bray distance metric. ARG abundance and environmental variables were passively fitted to community principle coordinates analysis data, and significance of fit was analyzed by means of permutation analysis ($n = 1000$).

Results and discussion

Spatial trends in ARG abundance

A central goal of this study was to characterize the spatial impact of Lausanne's WWTP discharge pipe on the contamination of Vidy Bay sediments with different ARGs. *sul* genes were detected at all and *tet* genes at several of the sampled locations, whereas *qnrA* was always below detection. ARG abundance generally decreased from *sul1* over *sul2*, *tet(w)*, *tet(M)* to *tet(B)* (Figure 2). The prevalence of ARGs in Vidy bay sediments thus reflects their abundance in Lausanne's wastewater: *qnrA* was detected in low amounts and only in hospital sewage, whereas

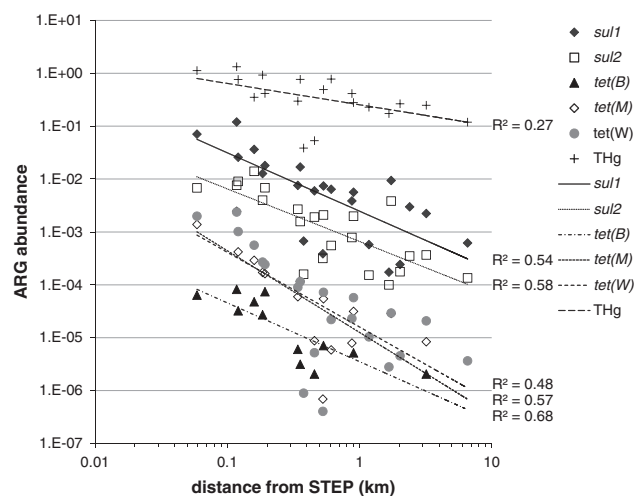


Figure 2 Log–log plot of ARG abundance (ARG copy numbers normalized to bacterial 16S rRNA gene copy numbers) versus distance from the STEP site. STEP was arbitrarily given a distance of $\frac{1}{2}$ the distance to the next closest site to allow it to be included in the analysis. Lines with R^2 values are for fitted functions of type $y = a \times x^b$.

sul and *tet* were more abundant in both WWTP influent and effluent samples, with *sul1* abundance always being the highest (Supplementary Figure S5). This abundance pattern may be related to the longer history of clinical application of sulfonamides and tetracyclines. *sul* and *tet* genes have previously been found to be quite abundant in sewage and receiving waters (Pruden *et al.*, 2006) and in livestock waste sludge (Zhang *et al.*, 2013). Plasmid-mediated quinolone resistance encoded by *qnrA* was first reported in 1998 (Martinez-Martinez *et al.*, 1998), which may explain its low abundance in Lausanne’s wastewater system. Other quinolone-resistance mechanisms were likewise infrequent, as shown by low colony counts on Ofloxacin- and Norfloxacin-amended agar plates inoculated with samples from Lausanne’s sewage and wastewater treatment system (Supplementary Figures S6 and S7).

For all the detected ARGs, highest gene concentrations and abundances occurred at sites in close proximity to the WWTP discharge (Figure 2), especially at sites STEP, EG2 and NC4 (Supplementary Figures S2 and S3). At site EG2, a maximum of 2.2×10^9 (*sul1*) and of 1.5×10^6 (*tet(B)*) ARG copies g^{-1} wwt were determined (Supplementary Figure S2). Abundance of *sul1* at this site reached 12% (Supplementary Figure S3). ARG concentration and abundance of both *sul* and *tet* genes were also high at sites NC1, NC2, NC3, NC5 and EG3 (Supplementary Figures S2 and S3), which are all located within 350 m of the STEP coordinate (Figure 1). Only *sul1* was also detected at comparatively high concentration and abundance at more remote sites (EG4, NC7, NC9 and NC14).

The relationship of ARGs with distance from the WWTP discharge pipe was not linear (Pearson’s

$r \sim -0.17$ for ARG concentration and $r \sim -0.36$ for ARG abundance, Supplementary Tables S4 and S5), but ARG abundance followed an exponential decay function (Figure 2). The decreasing trend appeared to be intact across the entire range of distances sampled. ARG concentration trends were similar as 16S rRNA gene copy numbers did not show a clear distance-related trend (Supplementary Figure S4). However, for each ARG, the R^2 was higher for abundance data compared with concentration data. A possible explanation would be sample-to-sample variations in PCR inhibition in spite of our best efforts to minimize such effects. We concluded that ARG abundance is slightly more robust for the purpose of our analysis. The R^2 for ARG abundance varied between 0.48 and 0.68, indicating that considerable amounts of variation are not explained by distance (Figure 2).

We therefore used spatial mapping to investigate whether deposition patterns deviate from a simple distance relationship, indicating directional transport (Figure 3 and Supplementary Figure S5). The maps clearly show the accumulation of ARGs around the WWTP discharge pipe. Designating a *sul1* abundance $> 1\%$ as ‘contaminated’, Figure 3a indicates an area of at least $\sim 0.3 \text{ km}^2$ has been impacted. All sampling points within this area are hereafter referred to as ARG-contaminated sediments.

ARG maps indicated a strong decrease in ARG abundance and concentration towards the deep lake (EG2 to EG9) and towards the south east (sampling locations EG14 and EG15). Ct values obtained for EG6, EG9, EG15 and the center of the lake (reference point SHL2) were either below the LOQ (*sul* genes) or even below LOD (*tet* genes). In contrast, our data indicated a less dramatic decrease in ARG levels along the transect following the shore line in south-western direction (NC5–NC13, Figures 2a–e and Figures 3a–e).

Finally, apart from site NC14 (close to the Chamberonne river—CHAM), samples taken close to shore (NC15, EG1) showed low levels of ARGs.

These observations indicate a directionality of the pollutant transport in the water column of Vidy Bay. Hydrodynamic transport in lakes is often complex. Currents are influenced by changing wind regimes, stratification and bathymetry and vary in direction and speed with depth (Wüest and Lorke, 2003; Righetti *et al.*, 2011). For Vidy Bay, it has recently been shown that near-shore gyres can occur (which enhance the retention time of water and pollutants in the bay) and that neither of two dominant wind regimes produce a consistent current pattern (Razmi *et al.*, 2013). However, westward shore-parallel currents can disrupt gyre formation and move water from near the STEP site to the water column above the DWP, with a dilution factor of about 100 (Razmi *et al.*, 2013). In previous studies, tracer released from the WWTP during holomixis reached surface waters at the DWP site (NC13) within 48 h, whereas no tracers were detected when thermal stratification precluded upwelling (Wildi and Rossi, 1997;

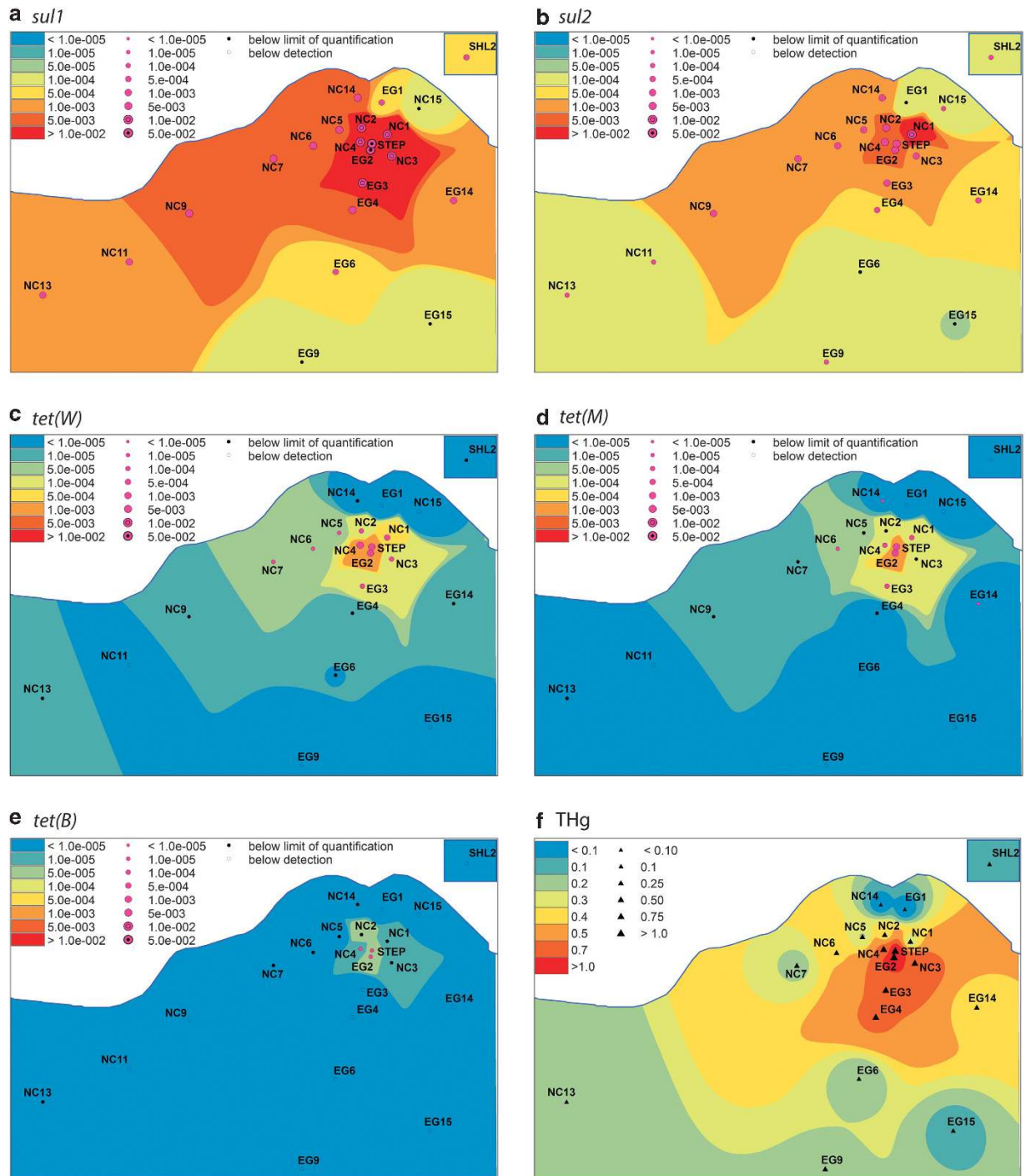


Figure 3 Spatial interpolation maps showing the distributions of ARG abundance (ARG copy numbers normalized to bacterial 16S rRNA gene copy numbers) and total mercury (THg, in mg kg^{-1}) in Vidy Bay sediments. (a) *sul1*, (b) *sul2*, (c) *tet(W)*, (d) *tet(M)*, (e) *tet(B)* and (f) THg.

Goldscheider *et al.*, 2007). In the case of particle-bound contaminants, the contamination record in sediments is expected to integrate over such temporally variable transport processes.

Contamination effects on the microbial community
Analysis of bacterial community similarity (Figure 4a) showed a clearly distinct community in sites close to the discharge pipe. This confirms

previous clone library-based observations from Vidy Bay (Haller *et al.*, 2011). The communities at the most strongly ARG-contaminated sites STEP and EG2 were highly similar to some untreated wastewater communities. Fitting of ARG abundances and environmental variables to the sediment community composition showed that these community changes correlated with indicators of contamination, such as ARG abundance, THg and total organic matter

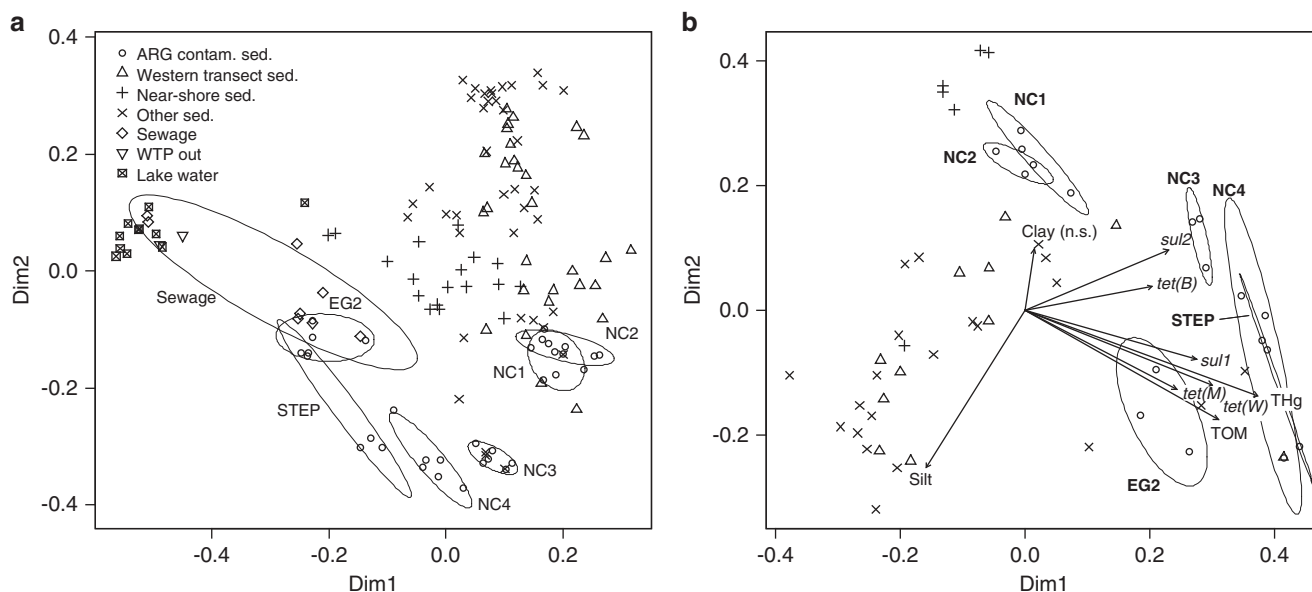


Figure 4 (a) Principle coordinates analysis of ARISA community profiles from sediment and water. Both biological and technical replicates are included. Dispersion ellipses are shown for sediment sampling sites near the WWTP discharge pipe and for untreated hospital and municipal wastewater (Sewage) (b) Principle coordinates analysis of ARISA community profiles from selected sediments with passively fitted environmental variables and ARG abundance. For this analysis, technical replicates were averaged, omitting peaks only present in one replicate. Samples were selected to match ARGs and sediment data availability. Symbols indicate samples grouped according to sample type and location. n.s., not significant ($P > 0.05$).

(Figure 4b). In contrast, particle composition parameters appeared either unrelated to the distinction between contaminated and uncontaminated sites (silt) or were not significantly correlated (clay, $P > 0.05$). Vectors fitted for *sul2* and *tet(B)* indicated a slightly different distribution within the microbial community compared with the other ARGs. Overall, community analysis indicated that changes in ARG abundance were related to changes in community composition, which can be explained by deposition of bacteria originating in wastewater. However, an impact of confounding factors, such as heavy metal pollution, cannot be ruled out by this analysis.

Comparison of ARGs and Hg contamination

The spatial trends in ARG distribution were compared with THg levels (Figures 2 and 3). Hg is a pollutant that is known to be released from Lausanne's WWTP (Loizeau *et al.*, 2004). Background concentrations in Lake Geneva sediments are $\sim 0.2 \text{ mg kg}^{-1}$. Although the chemistry and transport behavior of Hg is complex, particulate transport is dominant in most systems (Glass *et al.*, 1990; Wang *et al.*, 2004). Assuming that particle-associated bacteria or bacterial aggregates released from the WWTP are in turn primarily responsible for ARG deposition in the sediment, we expected a similar pollution pattern for THg and ARGs. However, ARG dynamics may be affected by bacterial mortality, for example, through predation, ultraviolet radiation or starvation. This could lead to a more restricted distribution compared with the heavy metal. On the other hand, released resistant

bacteria could proliferate or spread ARGs to the indigenous community via horizontal gene transfer. If the latter mechanisms dominate, a diffuse and more widespread pattern of ARG concentrations compared with THg would be expected. Differences as well as similarities in the spatial patterns of both contaminants may thus be informative.

Compared with previous studies (8 mg kg^{-1} sediment (Poté *et al.*, 2008)), a relatively low maximum THg concentration of 1.3 mg kg^{-1} sediment was measured during our survey (EG2, compare Figure 2f), indicating reduced recent deposition. We found elevated THg levels to be the highest around the STEP site but above background levels up to 600 m away in direction of the deep lake (EG4), 535 m away in the south-western direction (NC6) and 877 m away in the south-eastern direction (EG14, Figure 2f).

The similarity of the vectors of THg and ARG abundances fitted to community data in Figure 4b indicated a close link, which could either be deposition from a common source or because the heavy metal is a constraining factor of community composition. Nevertheless, there are notable differences in the spatial distribution, as shown by relatively low R^2 of linear regressions of THg and ARG concentration ($R^2 = 0.32\text{--}0.41$, $P < 0.05$, Supplementary Table S6) and abundance ($R^2 = 0.21\text{--}0.63$, $P < 0.01$, except for *sul2* with $P = 0.05$, Supplementary Table S7). The steeper slope of ARG abundance with distance compared with THg (Figure 2) indicates restrictions on ARG dissemination compared with THg. However, the south-western transect deviated from this general observation. Here, ARG levels decreased more

slowly, whereas sediments were classified as unimpacted in terms of THg (Figure 3f).

Finally, low levels of THg were determined at near-shore sites (0.04 and 0.05 mg kg⁻¹ for EG1 and NC14, respectively), most likely related to the comparatively large grain sizes and low organic matter content (Supplementary Table S8) of sediments deposited in this area by the Chamberonne and Flon rivers. In contrast, site NC14 near the Chamberonne river mouth showed elevated ARG levels (Figures 3a, b and f). Previous studies monitoring pollutant transport in the water column (Wildi and Rossi, 1997; Goldscheider *et al.*, 2007; Haldimann, 2009; Bonvin *et al.*, 2011) document that at least under certain circumstances site NC13 might be affected by contaminants released from the WWTP, including ARGs (Czekalski *et al.*, 2012). However, the Chamberonne river is known as a discontinuous source of fecal bacteria (Haldimann, 2009; Haller *et al.*, 2009), whereas its THg load is too low to explain elevated concentrations measured in sediments of Vidy Bay (Howa and Vernet, 1988). Our data show it to be a likely source of resistant bacteria and ARG contamination (compare Supplementary Figures S5, S6f and S7f). Low ARG levels detected at locations close to the shore further east (EG1 and NC15) would support the hypothesis that the Chamberonne plume is partly responsible for the extension of the ARG contamination in the south-western direction. This is also supported by current measurements in Vidy Bay in 2010, which revealed 45% of the currents moving west-southwest (Razmi *et al.*, 2013).

Direct and indirect causes of ARG contamination

Independently of transport, sediment properties might also influence the natural abundance of ARGs and the further fate of deposited allochthonous ARGs. Sediments containing a high fraction of clay minerals can bind DNA after cell death and protect it from degradation by DNase (Aardema *et al.*, 1983). In the sampled sediments, the proportion of clay was quite low (0.01–3.92%, Supplementary Table S8). ARGs have previously been detected in extracellular DNA in livestock waste sludge but were one to two orders of magnitude less frequent than the intracellular fraction, and both fractions showed similar dynamics (Zhang *et al.*, 2013). Therefore we assume that the detected ARGs reflect the ARGs in the viable bacterial biomass in our samples.

It has been demonstrated that heavy metals can cross- or co-select for ARGs (Berg *et al.*, 2010) as the same mechanisms often underlie both resistances, or resistance genes are located on the same mobile genetic element (Baker-Austin *et al.*, 2006; Knapp *et al.*, 2011). As sediments in Vidy Bay are contaminated not only with Hg but also with other heavy metals in excess of background levels (Poté *et al.*, 2008), it could be argued that the observed elevated ARG levels are caused by heavy metal pollution rather than bacterial deposition. In a study

carried out along a section of the Almendares River, which is affected by different urban and industrial contamination sources, good correlations were demonstrated for sediment ARGs (*tet* and β -lactamases) and copper levels (Graham *et al.*, 2010). Other heavy metals (Pb, Co, Zn) also partly correlated with ARGs, but neither Hg nor *sul* genes were considered in this study.

However, for the present study we conclude that the available evidence favors the hypothesis that the observed ARG levels are mainly a result of direct deposition of resistant bacteria rather than being due to co-selection by heavy metals: (a) The correlations of ARG levels with THg were relatively poor. The observed correlation can be explained by the parallel release of both types of contaminants from the WWTP. (b) The impact of the WWTP discharge on the deposition of fecal indicator bacteria and contrasting bacterial community composition has been shown previously (Haller *et al.*, 2011). (c) Our analysis of community similarity using ARISA (Figure 4) confirmed a distinct community composition of ARG-contaminated sediments in the vicinity of the discharge pipe that, furthermore, exhibits a high similarity to sewage communities (Figure 4a), supporting the notion that bacteria from these environments are deposited in the sediments. (d) A more rapid decrease of ARG abundance with distance compared with THg (Figure 2) is best explained by different dynamics during transport or deposition. Finally, (e) we found highly similar patterns of ARG abundance in wastewater/sewage and sediment (Supplementary Figure S4). Nevertheless, the role of the heavy metal contamination in Vidy Bay for selection or persistence of ARGs warrants further study.

Implications

We present detailed results on the spatial distribution of different ARGs in sediments of a freshwater lake that can be convincingly linked to the inflow of municipal wastewater. Our results demonstrate that the combination of qPCR and geostatistical tools is a suitable approach to quantitatively detect and assess the dissemination of ARGs from local sources. The release of wastewater into Vidy Bay has resulted in a high ARG accumulation in sediments in the vicinity of the contamination source, but a diffuse zone of elevated ARG concentrations extends further west towards the location of the drinking water pumping station, indicating that transport takes place over considerable distances. Uptake of ARGs via drinking water is considered a risk for human health as horizontal transfer to pathogens may occur. Treatment and quality control of waste and drinking water should be reconsidered with this in mind. In Switzerland and many other European countries, the majority of WWTPs operate without a final disinfection treatment, and many rely on basic biological treatment. As modernization of many of these facilities (including Lausanne's WWTP) is

imminent or already under way, it will be important to consider modern procedures for disinfection and contaminant removal that may be able to remove ARGs from waste and drinking water (Dodd, 2012). Effective ARGs and pollutant removal may help to break the cycle of ARG exchange between the human and the aquatic environment. The available technologies should be studied with regards to their effectiveness in reducing both the load of resistant bacteria and potentially transferable DNA. It should be kept in mind, however, that chemical disinfection methods may themselves cause an undesirable selection for antibiotic resistance. Even wastewater receiving elaborate treatment and disinfection has been shown to be a source of ARG contamination (LaPara *et al.*, 2011).

Conflict of Interest

The authors declare no conflict of interest.

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