A peer-reviewed version of this preprint was published in PeerJ on 3 January 2018.

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Madueño L, Paul C, Junier T, Bayrychenko Z, Filippidou S, Beck K, Greub G, Bürgmann H, Junier P. 2018. A historical legacy of antibiotic utilization on bacterial seed banks in sediments. PeerJ 6:e4197 https://doi.org/10.7717/peerj.4197

A historical legacy of antibiotic utilization on bacterial seed banks in sediments

Laura Madueño [1], **Christophe Paul** [1], **Thomas Junier** [2], **Zhanna Bayrychenko** [1], **Sevasti Filippidou** [1], **Karin Beck** [3], **Gilbert Greub** [4], **Helmut Bürgmann** [3], **Pilar Junier** Corresp. [1]

[1] Laboratory of Microbiology, Institute of Biology, University of Neuchatel, Neuchâtel, NE, Switzerland

[2] Vital-IT group, Swiss Institute of Bioinformatics, Lausanne, Switzerland

[3] Surface Waters - Research and Management, Swiss Federal Institute of Technology, Zurich, Kastanienbaum, Switzerland

[4] Institute of Microbiology, University Hospital of the University of Lausanne, Lausanne, Switzerland

Corresponding Author: Pilar Junier
Email address: pilar.junier@unine.ch

The introduction of antibiotics for both medical and non-medical purposes has had a positive effect in human welfare and agricultural output in the past century. However, there is also an important legacy in the use and disposal of antimicrobial agents in natural ecosystems. This historical legacy was investigated by quantifying two antibiotic resistance genes (ARG) conferring resistance to tetracycline (*tet*(W)) and sulfonamide (*sul*1) in bacterial seed bank DNA in sediments. The industrial introduction of antibiotics caused an abrupt increase in the total abundance of *tet*(W) and a steady increase in *sul*1. The abrupt change in *tet*(W) corresponded to an increase in relative abundance from ca. 1960 that peaked around 1976. This pattern of accumulation was highly correlated with the abundance of specific members of the seed bank community belonging to the Phylum Firmicutes. In contrast, the relative abundance of *sul*1 increased after 1976. This correlated with a taxonomically broad spectrum of bacteria, reflecting *sul*1 dissemination through horizontal gene transfer. The accumulation patterns of both ARGs correspond to the temporal scale of medical antibiotic use. Our results show that the bacterial seed bank can be used to look back at the historical usage of antibiotics and resistance prevalence.

A historical legacy of antibiotic utilization on bacterial seed banks in sediments

Short title: Antibacterial legacy in seed banks

Laura Madueño[1*], Christophe Paul[1*], Thomas Junier[1,2*], Zhanna Bayrychenko[1], Sevasti Filippidou[1], Karin Beck[3], Gilbert Greub[4], Helmut Bürgmann[3] and Pilar Junier[1&]

[1]Laboratory of Microbiology, Institute of Biology, University of Neuchâtel. Rue Emile-Argand 11, CH-2000, Neuchâtel, Switzerland

[2]Vital-IT group, Swiss Institute of Bioinformatics, Lausanne, Switzerland

[3]Eawag, Swiss Federal Institute of Aquatic Science and Technology, 6047 Kastanienbaum, Switzerland

[4]Institute of Microbiology, University of Lausanne and University Hospital (CHUV); Lausanne, Switzerland

[*]These authors contributed equally to this work.

[&]corresponding author: pilar.junier@unine.ch

Abstract

The introduction of antibiotics for both medical and non-medical purposes has had a positive effect in human welfare and agricultural output in the past century. However, there is also an important legacy in the use and disposal of antimicrobial agents in natural ecosystems. This historical legacy was investigated by quantifying two antibiotic resistance genes (ARG) conferring resistance to tetracycline (*tet*(W)) and sulfonamide (*sul*1) in bacterial seed bank DNA in sediments. The industrial introduction of antibiotics caused an abrupt increase in the total

abundance of *tet*(W) and a steady increase in *sul*1. The abrupt change in *tet*(W) corresponded to an increase in relative abundance from ca. 1960 that peaked around 1976. This pattern of accumulation was highly correlated with the abundance of specific members of the seed bank community belonging to the Phylum Firmicutes. In contrast, the relative abundance of *sul*1 increased after 1976. This correlated with a taxonomically broad spectrum of bacteria, reflecting *sul*1 dissemination through horizontal gene transfer. The accumulation patterns of both ARGs correspond to the temporal scale of medical antibiotic use. Our results show that the bacterial seed bank can be used to look back at the historical usage of antibiotics and resistance prevalence.

Introduction

The use of antibiotics to treat infectious diseases represents one of the major scientific achievements of the 20th century. Millions of lives have been saved since the introduction of antibiotics into general medical practice for the treatment of a large range of bacterial infections, as well as other medical procedures (Marti et al 2014). After the initial use of antibiotics in medicine, the utilization of antibiotics to increase agricultural productivity has become a common practice (Carlet et al 2011). Although the positive effect of the so-called antibiotic era on human welfare is not disputed, increased awareness of the risks posed by poor antibiotic stewardship mitigates this success. Nowadays, it is becoming clear that disposal of antibiotics in natural ecosystems can have far-reaching consequences. Recent studies on antibiotics and the emergence of resistance suggest that the function of antibiotics in nature cannot be explained solely within the paradigm of chemical weapon in which these compounds have been used since their industrial production (Aminov 2009, Aminov 2010). Instead, antibiotics and determinants of resistance are a fundamental component of the ecology of microbial ecosystems. Most of the

47 antibiotics used today are chemical derivatives of small bioactive molecules that perform a
48 multitude of functions in nature (Taylor et al 2011). Therefore, in evolutionary terms,
49 industrialized production, use, and disposal of antibiotics is a relatively recent phenomenon that
50 has presumably exerted a selective pressure for pathogens to acquire and further hone naturally
51 occurring antibiotic resistance systems (Taylor et al 2011). This phenomenon has given rise to
52 increasing rates of antibiotic resistance, a problem that threatens health care systems worldwide
53 (Wright 2010). Therefore, understanding the historical effect of antibiotic use on the natural
54 reservoirs of ARGs is essential to develop a management strategy to reduce current and future
55 risks.

56 ARGs were clearly present in microbial communities before the antibiotic era as shown by
57 phylogenetic analysis of genes conferring resistance to different classes of antibiotics (Aminov
58 and Mackie 2007). Given the presumed role of human activity in the levels of resistance in the
59 environment, one can thus expect an increasing abundance of such genes in the past century.
60 However, direct evidence for this is currently restricted to a limited number of studies. For
61 example, soil archives from two regions in Europe clearly demonstrate a link between the history
62 of antibiotic use and the increase in the abundance of various genes conferring resistance to a
63 large range of antibiotics (Graham et al 2016, Knapp et al 2010). Furthermore, the analysis of
64 soil records also demonstrated the interconnection between the medical and non-medical use of
65 antibiotics, as well as the effect of changes in policy towards a more strict stewardship (Graham
66 et al 2016).

67 Besides soils, aquatic ecosystems have been identified as a key ecological component driving the
68 emergence, spread, and persistence of antibiotic resistance (Taylor et al 2011). Lake sediments
69 are a major concern because they are a main environmental end-point not only for bacteria, but

also for ARGs and antimicrobial agents (Kümmerer 2009). The high numbers of cells in sediments made resuspended sediment material a highly likely source of resistance determinants. At the same time, lake sediments are natural environmental archives. Thus, the study of the sedimentary record might provide insights into the historical legacy of the antibiotic era and the accumulation of ARG in the environment. Attempts to use DNA extracted from sediments to investigate antibiotic resistance in aquatic systems have been made (Thevenon et al 2012), but suffer from uncertainty regarding the preservation of the environmental signal in the sediments. Sediment microbial communities are strongly shaped by the redox gradients experienced during early diagenesis, and it is therefore unclear how much of the originally resistant community, or of their resistance determinants, is preserved in deeper sediment layers, or how this relationship is affected by environmental factors. The use of microbial seed banks preserved in the sedimentary record as a proxy offers a likely solution to these problems.

The seed bank can be broadly defined as a reservoir of dormant cells that can potentially be resuscitated under favorable environmental conditions (Lennon and Jones 2011). One of the defining features of dormant cells is their reduced metabolic activity (Driks 2002), decreasing the uncertainty generated by environmental changes during sediment diagenesis (Vuillemin et al 2016). In addition, dormant cells are more resistant to degradation than their actively growing counterparts (Abecasis et al 2013). We have used the latter property to develop a specific extraction method to enrich DNA from spores as an example of dormant cell forms (Wunderlin et al 2014b, Wunderlin et al 2016). With this approach we have previously shown that one particular group of bacteria capable of dormancy (endospore-forming Firmicutes) can be used as paleoecological biomarkers of the impact of lake eutrophication on microbial communities in sediments (Wunderlin et al 2014a). Using the same selective method we investigated if the

historical antibiotic usage has affected the levels of ARG found in the natural seed bank bacterial community.

Material and Methods

Site description and sampling

A sediment core was retrieved with a gravity corer (UWITEC, Mondstein, Au) in August 2011 in an inactive canyon (C1) on the eastern side of the Rhone delta in Lake Geneva (Switzerland) (CAN01, coordinates 559901-139859, 79 m depth, 105 cm). This core has previously been dated (^{137}Cs and magnetic susceptibility dating) and validated for paleoecology (Wunderlin et al 2014a).

DNA extraction

DNA from the seed bank was obtained using an indirect extraction method. The extraction of cells from sediments was performed as previously described (Wunderlin et al 2013). The cells extracted from 3 g of wet sediment were filtered onto two different 0.2 um pore-size nitrocellulose filters (Merck Millipore, Darmstadt, Germany). A treatment to separate seed bank from vegetative cells was performed on the biomass collected on nitrocellulose filters, as previously described (Wunderlin et al 2014b, Wunderlin et al 2016). One filter (1.5 grams of sediment) per sample was used for the treatment. The first step consisted of the lysis of vegetative cells by heat, enzymatic agents (lysozyme) and chemicals (Tris-EDTA, NaOH, SDS). Further DNase digestion was used to destroy any traces of free DNA. DNA was extracted from the pre-treated filters using a modified protocol with the FastDNA®SPIN kit for soil (MP Biomedicals, USA)(Wunderlin et al 2013), in which the lysing matrix was submitted to two successive bead-beating steps. Supernatants from each bead-beating step were treated separately

downstream according to manufacturer's instructions. DNA extracts were pooled by precipitation with 0.3 M Na-acetate and ethanol (99 %), stored at -20°C overnight and centrifuged for 1h at 21. 460 x g and 4°C. Supernatant was removed and the pellet was washed with 1 volume of 70% ethanol and centrifuged for 30 min at 21.460 x g and 4°C. Supernatant was removed and the residual ethanol was allowed to evaporate at room temperature. DNA was re-suspended in 50 µl of PCR-grade water. Total DNA was quantified using Qubit® dsDNA HS Assay Kit on a Qubit® 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA).

Quantitative PCR on *tet*(W) and sul1 **genes**

Quantitative Taqman®-PCR on sul1 and *tet*(W) genes was performed in 384-well plates using a LightCycler®480 Instrument II (Roche, Switzerland). For sul1, the primers used were qSUL653f and qSUL719r with tpSUL1 probe (Heuer and Smalla 2007). The reaction mix for sul1 consisted of 2 µL of DNA template (between 0.08 and 1.39 ng/µL), 0.025 µM of each primer, 0.25 µM of TaqMan probe and 1 x TaqMan®Fast Universal PCR Master Mix (Applied Biosystems, USA). Total reaction volume of 10 µL was reached with PCR-grade water. For *tet*(W), the primers used were tetW-F and tetW-R with tetW-S probe (Walsh et al 2011). The reaction mix for *tet*(W) consisted of 2 µL of DNA template, 0.025 µM of each primer, 0.1 µM of TaqMan probe and 1 x TaqMan®Fast Universal PCR Master Mix (Applied Biosystems, USA). Total reaction volume of 10 µL was reached with PCR-grade water. The qPCR program was the same for both genes and started with a hold at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 s and annealing/elongation at 60°C for 1 min. The qPCR assays were performed in technical triplicates on samples, standards and negative controls. The negative controls consisted of PCR blanks with only the reaction mix and of PCR blanks containing the mix and 2 µL of PCR-grade water. Standard curves were prepared from serial 10-fold dilutions of plasmid DNA containing

the respective target gene in a range of 5 x 10^7 to 50 gene copies. For sul1, control plasmids and standard curves were prepared as previously described (Heuer and Smalla 2007). For *tet*(W), standard curves were prepared as previously described (Walsh et al 2011). The effect of inhibitors on amplification was tested for all the samples and for both genes. All samples were spiked with 10^4 copies of plasmid DNA containing the *tet*(W) or the sul1 gene and amplified together with the same set of non-spiked samples and control DNA and the results indicated that inhibition was negligible.

Sequencing and data analysis

Purified DNA extracts were sent to Fasteris (Geneva, Switzerland) for 16S rRNA amplicon sequencing using Illumina MiSeq platform (Illumina, San Diego, USA), generating 250 bp paired-end reads. The hypervariable V3-V4 region was targeted using universal primers Bakt_341F (5'-CCTACGGGNGGCWGCAG-3') and Bakt_805R (5'-GACTACHVGGGTATCTAATCC-3') (Herlemann et al 2011). Analysis of the dataset was made using Mothur (Schloss et al 2009) following the standard MiSeq SOP(Kozich et al 2013). The SILVA reference database (Quast et al 2013) was used for the alignment of amplicons and the taxonomic assignment of representative OTUs. After quality filtering and removal of chimeras, a total of 2'837'393 amplicons was obtained (625'339 unique sequences). Singletons were removed prior to the clustering into OTUs. The number of singletons in the dataset was 560'158. Clustering of the 2'277'235 remaining sequences (65'181 unique sequences) was made using a threshold of 97% identity. Finally, 11'802 OTUs constitute the dataset. The generated datasets were submitted to NCBI under the Bioproject accession number PRJNA396276.

Statistical and multivariate analyses

Community and statistical analyses were performed using R version 3.4.0 (R Team 2014) and the *phyloseq* and *vegan* packages (McMurdie and Holmes 2013, Oksanen et al 2017). Pairwise correlations between OTUs relative abundance and ARGs frequency were calculated using Spearman's rank correlation coefficient. Seed bank community was analyzed by principal coordinates analysis (PCoA), based on Bray-Curtis dissimilarity and Hellinger transformation of the OTUs table (community matrix). Environmental parameters and ARGs abundance/frequency were standardized and passively fitted to the ordination. Only significant parameters were displayed ($p<0.05$).

Results

Quantification of ARGs in seed bank communities from sediment samples

Seed bank DNA was extracted from a sediment core previously validated for paleoecology covering approximately the last hundred years of sediment accumulation in Lake Geneva (Wunderlin et al 2014a). ARG in seed bank DNA was measured by quantifying the number of copies of genes conferring resistance to tetracycline (*tet*(W) gene) and sulfonamide (*sul*1 gene), two commonly reported antibiotics detected in environmental settings (Davies and Davies 2010). The detection of ARGs in the seed bank DNA changed beginning in 1960 (*tet*(W)) and 1970 (*sul*1). However, the accumulation pattern was different for the two ARGs. In the case of *tet*(W), the total abundance of the gene (copies/g of sediment) increased by an order of magnitude since 1965 compared to the values obtained from 1920 to 1960 (Supplementary Figure 1). Moreover, the relative abundance of this ARG (gene copies/ng of DNA) in the seed bank DNA increased from 1961 to 1975 (Figure 1). In the case of *sul*1, a steady increase of this ARG abundance was observed after 1970 (Supplementary Figure 1). The relative abundance of *sul*1 in seed bank

184 DNA increased from the same period, followed by a decline and a more recent increase after the
185 year ca. 2000 (Figure 1). The specific timeframe in which enrichment in ARG counts per ng of
186 DNA was observed concerned mainly the seed bank DNA, as opposed to the total bacterial
187 community. In addition, we could detect ARGs using a lower initial concentration of DNA for
188 the seed bank community (2 ng of DNA) compared to the total community (10-15 ng of DNA).
189 This further suggests a preferential enrichment of ARGs in seed bank bacteria compared to the
190 overall environmental background.
191
192 **Characterization of the seed bank communities**
193 Previous studies in Lake Geneva have shown a dramatic effect of human activity on the
194 nutritional status of the lake. The lake became eutrophic between 1954 and 1986, and this
195 modified the proportion of some members of the bacterial community in sediments (Wunderlin
196 et al 2014a). Eutrophication is partly related to the same human activities that also shaped the
197 antibiotic era (for example, increased agricultural and livestock output and population pressure).
198 Since changes in microbial community composition as well as the spread of ARG within
199 populations can influence the record of antibiotic resistance, it was important to analyze seed
200 bank community composition alongside ARG quantification. Representatives of six major
201 bacterial phyla (Proteobacteria, Firmicutes, Actinobacteria, Planctomycetes, Chlamydiae, and
202 Chloroflexi) were the main components of the bacterial seed bank community in sediments
203 (Supplementary Figure 2; Figure 2A). The overall community analysis revealed similarities in
204 the community composition in samples with higher relative abundance of either *tet*(W) or *sul*1
205 (Figure 2B). For the former, a significant contribution of OTUs associated to the Phylum

206 Firmicutes was observed, while in the case of *sul*1 no particular bacterial group was correlated
207 with increased accumulation.
208 In order to understand more clearly the relationship between ARG enrichment and seed bank
209 bacterial community, we next studied if the relative abundance of certain OTUs was correlated
210 with ARG levels. For this, we calculated the correlation coefficient between the relative
211 abundance of each OTU and the ARG relative abundance at different depths. Correlation
212 coefficients were plotted as a continuum to analyze the overall response of the community
213 (Figure 3A). In the case of *tet*(W) most of the non-Firmicutes seed bank community was not
214 correlated with increased ARG relative abundance over time (most correlation coefficients were
215 close to 0; Figure 3A; dashed line). However, when the analysis is made only for representatives
216 of the Phylum Firmicutes, the distribution shifted significantly towards positive correlations
217 (comparison of the distribution for the total and Firmicutes communities; $t = 16.52$, $df = 6171.6$,
218 p-value < 2.2e-16; Figure 3A; solid line). This analysis confirmed the results of the total
219 community analysis (Figure 2B). We investigated further the ten most positively correlated
220 OTUs. Nine out of the ten operational taxonomic units (OTUs) positively correlated with *tet*(W)
221 relative abundance belong to Firmicutes (Table 1). The origin and ecology of bacteria related to
222 those OTUs suggests an equal contribution of bacteria from an environmental origin, mainly
223 cellulose-degrading anaerobic bacteria such as *Anaerobacterium* (Horino et al 2014) (OTU00093
224 and OTU00528), *Clostridium* (Hernandez-Eugenio et al 2002, Miller et al 2011, Zhilina et al
225 2005) (OTU00262, OTU00084, and OTU02280), and *Acetivibrio* (Patel et al 1980)
226 (OTU00908); and from human (or animal) intestinal origin such as *Ruminoccous* (Cann et al
227 2016, Chassard et al 2012, Crost et al 2016) (OTU01612 and OTU01577). The OTUs positively
228 correlated to *tet*(W) represented a minor fraction of the bacterial seed bank community even for

229 those samples with the highest ARG abundance (relative OTU abundance not higher than 5%;
230 Figure 3B).
231 The same analysis performed on *sul*1 showed a larger fraction of the community positively
232 correlated to relative ARG abundance (Figure 3A), but in contrast to *tet*(W) this is not
233 specifically significant for Firmicutes only. Instead, the 10 most positively correlated OTUs
234 belonged to diverse phylogenetic groups (Actinobacteria, Chloroflexi, Firmicutes, Proteobacteria,
235 Verrucomicrobia, and Planctomycetes) (Table 1). OTUs correlated positively with *sul*1
236 abundance represented only minor fractions of the seed bank community (Figure 3A).
237 Interestingly, the correlation coefficients are higher for *tet*(W) than for *sul*1, suggesting a
238 stronger relationship of particular OTUs with the former.
239 Even though the analysis of the total community already suggests that the effect of increased
240 relative abundance of ARG is independent from the generalized effect of eutrophication, we
241 performed the same correlation analysis between relative OTU abundance and iron and
242 manganese concentrations in sediments. Iron and manganese can be used as a proxy for redox
243 conditions in the water column (Corella et al 2012, Koinig et al 2003) and their concentration
244 correlates with eutrophication in Lake Geneva (Wunderlin et al 2014a). The results show no
245 overlap between the overall effect of eutrophication and the specific effect of ARG abundance in
246 terms of the most correlated OTUs (Supplementary Figure 3).
247

248 **Discussion**

249 Lake Geneva is one of the largest lakes in Europe and constitutes a major reservoir of drinking
250 water. The composition of bacterial communities (Haller et al 2011, Sauvain et al 2014), as well
251 as the presence of toxic metals (Pote et al 2008), micropollutants (Bonvin et al 2011), and ARGs

(Czekalski et al 2012, Czekalski et al 2014, Devarajan et al 2015), has been monitored regularly in its water column and sediments. All these studies have demonstrated the role of human activity in the transfer of contaminants (including antibiotics) into sediments. All these preliminary studies made of Lake Geneva an ideal model system to validate the use of the seed bank bacterial community as a proxy to the effect of the historical use of antibiotics on the abundance of ARG in the environment. Our results show that studying the bacterial seed bank community in sediments of Lake Geneva shows the historical increase in ARG abundance. There was a clear link between seed bank taxonomy and accumulation of *tet*(W). This taxonomy-specific effect has been well documented in the case of tetracycline (Roberts and Schwarz 2016). Tetracycline is a class of broad-spectrum antibiotics active against a wide range of Gram-positive and Gram-negative bacteria, including some atypical pathogens such as *Mycoplasma* and *Chlamydia*, and even eukaryotic parasites. This antibiotic class was isolated from *Streptomyces* spp. between 1947 and 1950, constituting one of the earliest classes of antibiotics described (Roberts and Schwarz 2016). In the USA, tetracycline became extensively used in production of livestock between 1950s and 1970s and remains today the second most commonly used antibiotic in agriculture (Roberts and Schwarz 2016). The situation in Switzerland is similar, according to a recent report from the Swiss Federal Office of Public Health indicating that tetracycline (together with penicillin) is the second most sold antibiotic product, after sulfonamides (FOPH 2016). In Switzerland, the current use of tetracycline is mainly restricted to non-medical applications, with a reported consumption below 1% in hospitals (according to data covering the period from 2004 to 2015) and close to 11% in outpatient settings (FOPH 2016). In Switzerland the principal medical use of tetracycline was reported for the period of 1955 to 1970 (Table 2), but has since reduced dramatically following the use of amoxicillin-clavulanate for

skin and soft-tissue infections and the increased use of cotromixazole (a combination of sulfonamides and trimethoprim) for uncomplicated urinary tract infections, which represent the two most common bacterial infections encountered in outpatient clinics and private medical practice. This medical historical use fits well with the observed peak of relative accumulation of *tet*(W) in the seed bank DNA in the late 1970s, and would suggest a primarily medical origin to this ARG for this period of time.

Tetracycline binds to the elongating ribosome, affecting translation, and therefore resistance can be acquired through diverse mechanisms (Davies and Davies 2010, Roberts and Schwarz 2016). *tet*(W) confers resistance through ribosomal protection and although the ancestral source of the gene is unknown, it has been reported in both Gram-positive and Gram-negative bacteria (Roberts and Schwarz 2016). Even though our analysis cannot determine the origin of *tet*(W) in sediments, it clearly suggests that the accumulation of this gene in the seed bank during the medical use of this antibiotic is highly correlated with changes in the abundance of Firmicutes. One potential explanation for the link between medical use of tetracycline and *tet*(W) in Fimicutes is the fact that the human gut microbiome can serve as a reservoir of ARGs, and in particular to genes conferring resistance to tetracycline (de Vries et al 2011, van Schaik 2015). A recent analysis of the human gut microbiome suggests that Firmicutes are highly prevalent (Browne et al 2016, Dethlefsen et al 2007). More importantly, a recent study suggests that sporulation is a widespread characteristic of the human microbiome (Browne et al 2016), and it is precisely these dormant forms that can contribute to the seed bank in human-impacted ecosystems. However, linking *tet*(W) abundance and the human microbiome must not be seen as a confirmation of the relationship between medical antibiotic use and increase of ARGs levels in the environment. For example, a recent study monitoring the effect of tetracycline on the

298 performance of anaerobic digestors used in wastewater treatment has shown a highly significant
299 increase in the relative abundance of spore-forming Firmicutes after treatment with a
300 concentration of 20 mg/L of tetracycline (Xiong et al 2017). Overall the data suggest that
301 antibiotics such as tetracycline can select for specific groups of Firmicutes.
302 The industrial introduction of sulfonamide was an entirely different effect to that of tetracycline.
303 Sulfonamide drugs were also among the earliest antibiotics discovered. However, in contrast to
304 tetracycline, sulfonamide and its derivatives were obtained by systematic screening of
305 chemically synthesized compounds. The legacy of mass production of sulfonamide is reflected in
306 one of the most broadly disseminated case of drug resistance, both in terms of prevalence and
307 taxonomy (Aminov 2010). Resistance to this class of antibiotic is almost universally associated
308 to genetic mobile elements that confer a fitness advantage to the receptor bacteria as shown in
309 the case of non-pathogenic *Escherichia coli* (Enne et al 2004). The abundance of *sul*1 may thus
310 be indicative of a dissemination trend of certain widespread mobile genetic elements (e.g. class-1
311 integrons) (Gillings 2014, Skold 1976, Skold 2000) that may well carry other resistance elements.
312 Horizontal gene transfer mediated by mobile genetic elements is considered a major pathway of
313 ARG dissemination in aquatic environments (Berglund 2015). This particular mechanism of
314 ARG dissemination overcomes taxonomic barriers, probably explaining the wide taxonomic
315 spectrum of bacterial seed bank groups related to *sul*1 quantification in the sediments.
316 Changes in guidelines to reduce usage of penicillin derivatives (such as co-amoxicillin) for
317 uncomplicated urinary tract infection in favor of cotrimoxazole (Sulfamethoxazol-Trimethoprim
318 combination) may partially explain the common occurrence of *sul*1 resistance gene in the seed
319 bank DNA especially after 2005 (Table 2). At this time medical guidelines changed given the
320 high rate of resistance of *E. coli* (90% of the etiology of cystitis in healthy adult female humans)

to penicillin derivatives, leading to the reintroduction of sulfonamides. Indeed, the resistance rate of *E coli* to amoxicillin and to amoxicillin-clavulanate respectively reached 52% and 23% of the isolates tested at the Lausanne University Hospital Diagnostic Laboratory in 2016 (4581 strains).

Conclusions

Previous studies on the historical legacy of the antibiotic era have come to contradictory conclusions. On the one hand, they show the recent effect of human activity on ARGs in the environments (Graham et al 2016, Knapp et al 2010, Thevenon et al 2012), and suggest that reducing non-therapeutic antibiotic use can reduce some of the environmental ARG legacy. On the other hand, the results show that this is not universally applicable to all antibiotic classes and that policies intended to reduce non-therapeutic use can have undesirable consequences (Graham et al 2016). Results for the accumulation of beta-lactamase genes in soils suggest that soil accumulation reflected a broader expansion of antibiotic use across society, implying that development of resistance in clinical and agricultural systems is mutually influential (Graham et al 2016). Our data adds valuable information to the debate regarding the long-term effect of the antibiotic era as we show that ARGs also affect a fraction of the microbial community that will certainly outlast many of these policies: the seed bank bacterial community. This opens up a new debate, concerning the potential long-term effect of these dormant, persistent ARG-contaminated cellular structures and their potential for further spreading of ARGs in the environment. Importantly however, we here by provide a proof of concept for a new way to study the historical development of resistance that is applicable to many geographic regions and resistance determinants and that does not rely on human archiving of environmental samples.

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Acknowledgments

Swiss National Science Foundation (Interdisciplinary grant CR23I2_162810) for the funding of SF and TJ.

Conflict of interest

The authors declare no conflict of interest or competing financial interest.

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Tables & Figures legends

Table 1. Correlation analysis between individual OTUs and relative abundance of *tet*(W) and *sul1*. Top 10 most positively and negatively correlated OTUs. For *tet*(W) gene, mostly OTUs belonging to Firmicutes have been correlated to *tet*(W) abundance. In contrast, for *sul1,* OTUs correlated to *sul1* abundance belong to many phyla.

Table 2. Summary of antibiotic discovery, use and year in which resistance was documented. Temporal scale showing the respective period when a new antibiotic has been discovered, main period of clinical usage and the approximate year when a first resistance to that compound has been documented. The table is partially adapted from multiple sources(Clatworthy et al 2007, Torok et al 2009, van Hoek et al 2011), including national and international guidelines, as well as personal communication with Swiss and French doctors.

Figure 1. Tetracycline and Sulfonamide resistance in total bacterial community and in the seed bank over time. Relative abundance (gene copies/ng of extracted DNA) of two genes conferring resistance to the antibiotics tetracycline (*tet*(W)) and sulfonamide (*sul*1) in sediment samples covering the period between 1920 and 2010 in Lake Geneva, Switzerland. Quantification was made in DNA extracted from the seed bank (SB DNA) and total microbial community (total DNA).

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Correlation analysis between individual OTUs and relative abundance of *tet*(W) and sul1.

Top 10 most positively and negatively correlated OTUs. For *tet*(W) gene, mostly OTUs belonging to Firmicutes have been correlated to *tet*(W) abundance. In contrast, for *sul1,* OTUs correlated to *sul1* abundance belong to many phyla.

Tables

Table 1. Correlation analysis between individual OTUs and relative abundance of *tet*(W) and sul1. Top 10 most positively and negatively correlated OTUs.

Gene	OTU	Phylum	Genus	Correlation coefficient
***tet*(W)**	Otu00093	Firmicutes	*Anaerobacterium*	0.7890
	Otu01612	Firmicutes	*Lachnoclostridium*	0.7391
	Otu00262	Firmicutes	Clostridiaceae 1 unclassified	0.7136
	Otu00528	Firmicutes	*Clostridium* unclassified	0.6990
	Otu01577	Firmicutes	*Ruminococcus* 1	0.6791
	Otu00084	Firmicutes	Ruminococcacea unclassified	0.6722
	Otu00908	Firmicutes	Ruminococcacea unclassified	0.6684
	Otu02280	Firmicutes	*Epulopiscium*	0.6684
	Out01131	Verrucomicrobia	Verrucomicrobiales unclassified	0.6659
	Otu00529	Firmicutes	*Geobacillus*	0.6652
***sul*1**	Otu00318	Actinobacteria	*Mycobacterium*	0.6656
	Otu00382	Chloroflexi	Caldilineaceae unclassified	0.6517
	Otu00975	Firmicutes	*Ruminiclostridium* 1	0.6479
	Otu03004	Firmicutes	*Symbiobacterium*	0.6341
	Otu03302	Actinobacteria	Actinobacteria unclassified	0.6195
	Otu00155	Proteobacteria	*Hypomicrobium*	0.6176
	Otu00604	Verrucomicrobia	Verrucomicrobia unclassified	0.6170
	Otu00853	Acidobacteria	Subgroup 6 unclassified	0.6103
	Otu02777	Actinobacteria	*Tessaracoccus*	0.6095
	Otu01652	Planctoymcetes	Plactomycetaceae unclassified	0.6092

Table 2(on next page)

Summary of antibiotic discovery, use and year in which resistance was documented.

Temporal scale showing the respective period when a new antibiotic has been discovered, main period of clinical usage and the approximate year when a first resistance to that compound has been documented. The table is partially adapted from multiple sources (Clatworthy et al 2007, Torok et al 2009, van Hoek et al 2011) , including national and international guidelines, as well as personal communication with Swiss and French doctors.

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Antibiotics (class)	Discovery (year)	Period of usage[a]	Resistance (year)[b]
Sulfonamides	**1930**	**1940-1960** **1970-1985**[c] **2005-2017**	**1940**
Tetracycline	**1948**	**1955-1970**	**1953**
Penicillin	1929	1930-1970 2005-2017[d]	1947
Methicillin	1960	1960-2017	1962
Ampicillin	1962	1965-2017[e]	1974
Cephalosporins	1960-1970[f]	1965-2017[g]	1970
Vancomycine	1957	1970-1995 2000-2017[h]	1988
Streptomycin (Aminoglycosides)	1943	1946-1960 1980-2000	1958
Chloramphenicol	1947	1950-1970	1958
Erythromycin (Macrolides)	1952	1995-2010[i]	1988
Norfloxacin (Quinolones)	1979	1986-1995[j]	1981

Ciprofloxacin (Quinolones)	1987	1990-2005[k]	1988
Linezolid	2000	2010-2015	2004
Daptomycin	2004	2012-2017	2005
Clindamycin (Lincosamides)	1960	1960-1975[l]	1964

[a]Estimates made for Europe; in some sub-Saharan countries, due to the difficulties of access some antibiotics (such as chloramphenicol and streptomycin) are still largely in use.

[b]Approximate date, mainly adapted from(Clatworthy et al 2007, van Hoek et al 2011). The year of resistance documentation is often much earlier than the year of the description of the mechanism leading to a resistance phenotype. For example for aminoglycosides, the first identified resistance mechanism was the decreased permeability, which was initially described only *in vitro*.

[c]With the availability of cotrimoxazole since 1968, there has been an increased use of cotrimoxazole until about 1985, when 2nd generation quinolones (such as ciprofloxacine) have been largely available.

[d]Recent increase in use of penicillin instead of methicillin or cephalosporins for susceptible strains in order to attempt to reduce selection pressure due to overuse of antibiotics.

[e]Including its use in combination with clavulanate.

[f]Successive discovery of first- second- and third generation of cephalosporins.

[g]There is still a wide use of cephalosporins in Switzerland nowadays, mainly ceftriaxone for the treatment of severe infections due to Gram negative bacteremia, including *E. coli* bacteremia, which represents the most common cause of bacteremia (mainly in the setting of urosepsis).

[h]After a first wide use of vancomycin in initial empirical therapy, especially for severe infections such as endocarditis, bacteremia and fever in neutropenic subjects, the use of vancomycin slightly decreased due to concern about emerging resistance in enterococci; use of vancomycin then again increased due to surge in prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) and then due to common vancomycin-resistant enterococci (VRE).

[i]Larger usage of macrolides when clarithromycin and azithromycin have been made available, especially for the empirical treatment of lower respiratory tract infections (in combination with cephalosporins, when severe)

[j]Norfloxacin was largely replaced by ciprofloxacin as first line treatment for urinary tract infection from 1995 onwards, due to concern regarding antibiotic resistance and improved efficacy of ciprofloxacin for complicated urinary tract infections; in 2008, the European Medicines Agency recommended to avoid using oral norfloxacin for treatment of urinary infections (http://www.docguide.com/emea-restricts-use-oral-norfloxacin-drugs-utis)

[k]Ciprofloxacin largely replaced norfloxacin for urinary tract infection from 1995 to 2005; in addition from 1990 to 2000, ciprofloxacin was largely used for the empirical treatment of lower respiratory tract infections (LRTI); then due to increased concern about resistance, cotrimoxazole was proposed as first-line empirical antibiotic treatment for uncomplicated urinary tract infection and macrolides replaced quinolones in the treatment of LRTI. This change for a decreasing usage of quinolones was also triggered by the decreased rate of susceptible Gram-negative bacilli to quinolones, which decreased from about 90% in 1990 to about 65-70% in 2000 in USA and in Europe.

[l]Mainly used to treat staphylococcal infections from 1965 to 1975; however, its usage has declined much due to documentation of resistance and due to possible increased risk of post-

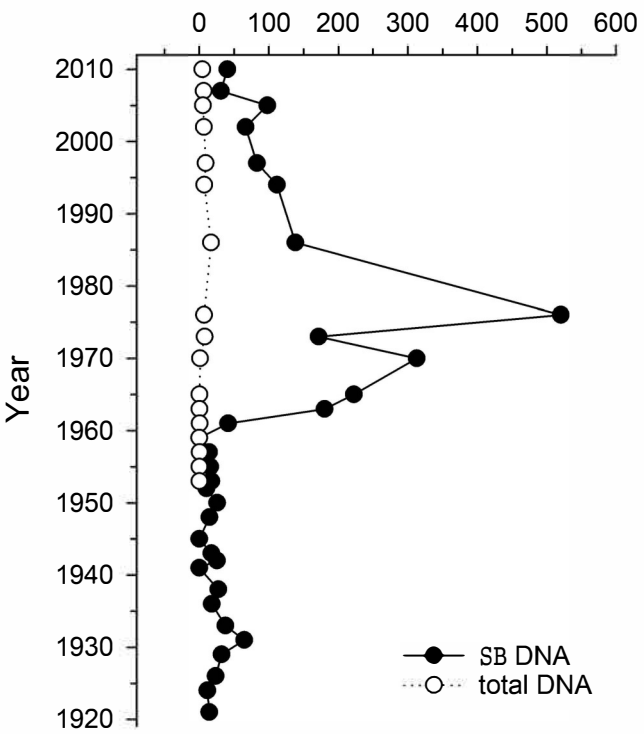
antibiotic colitis due the broad antimicrobial effect of clindamycin on anaerobes, which constitute more than 90% of the intestinal microbiota.

Figure 1(on next page)

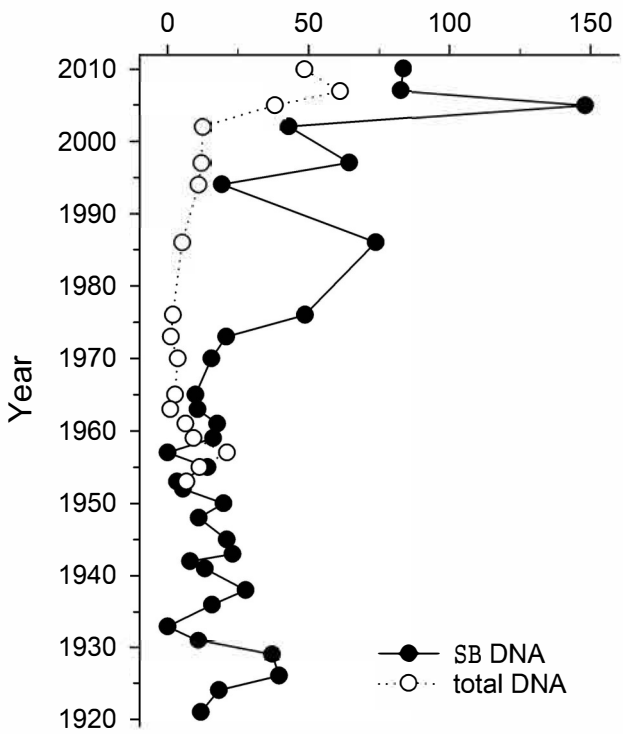
Tetracycline and Sulfonamide resistance in total bacterial community and in the seed bank over time.

Relative abundance (gene copies/ng of extracted DNA) of two genes conferring resistance to the antibiotics tetracycline (*tet*(W)) and sulfonamide (*sul*1) in sediment samples covering the period between 1920 and 2010 in Lake Geneva, Switzerland. Quantification was made in DNA extracted from the seed bank (SB DNA) and total microbial community (total DNA).

tet(W)



*sul*1



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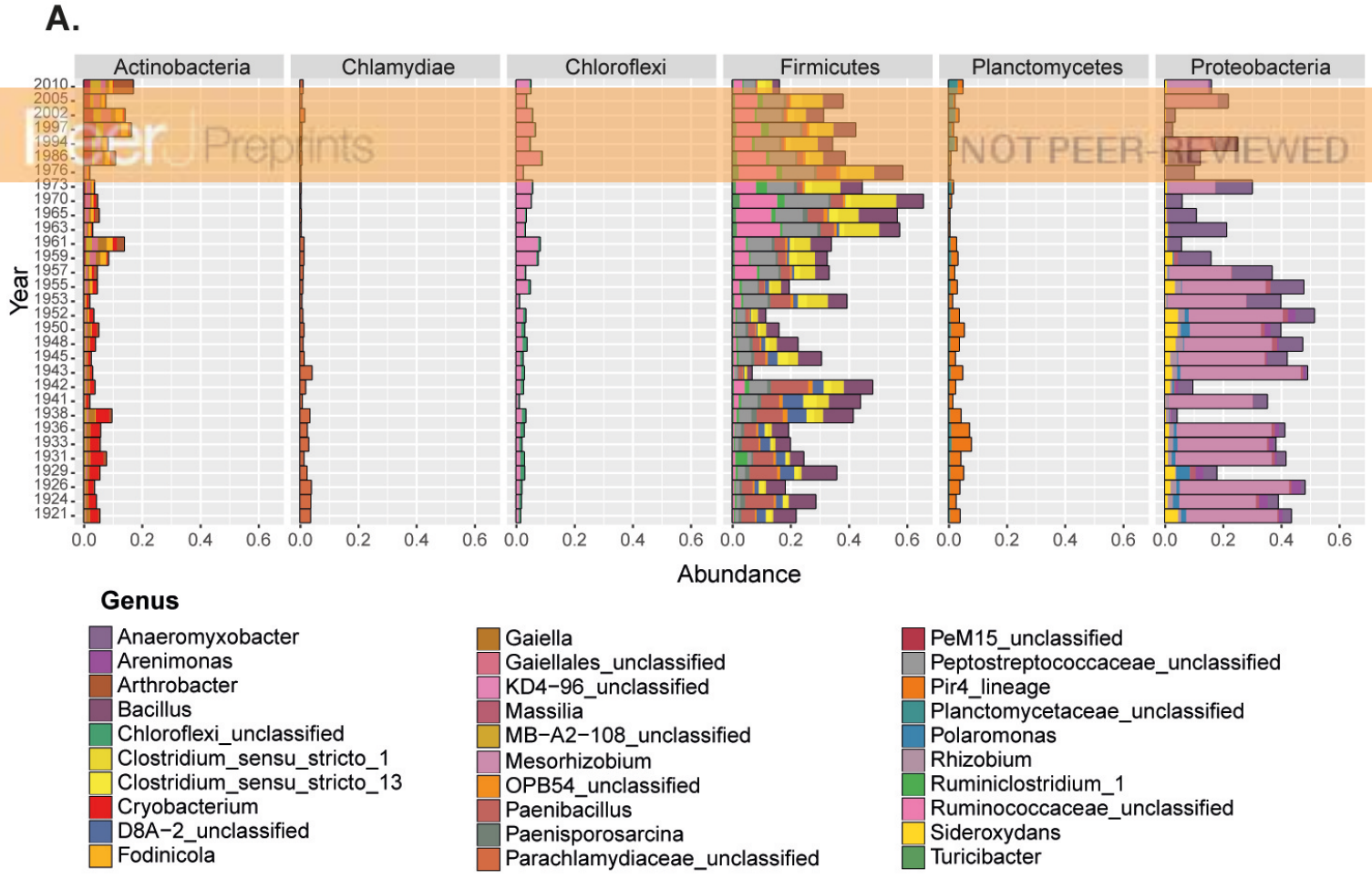

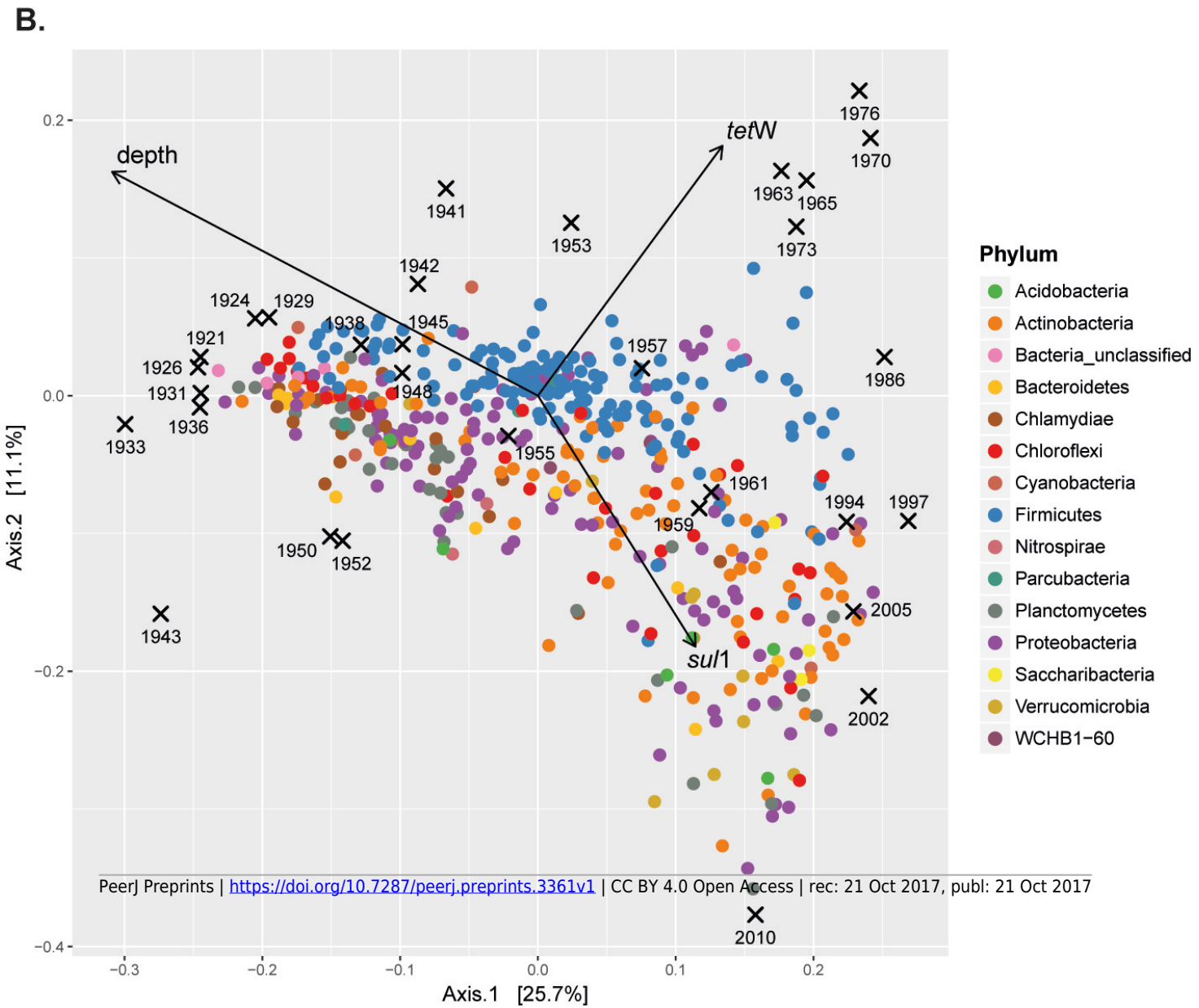

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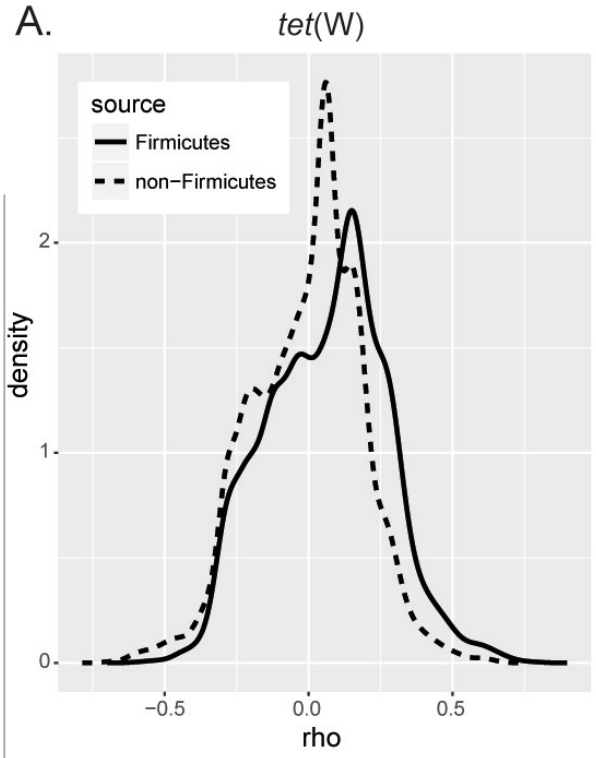

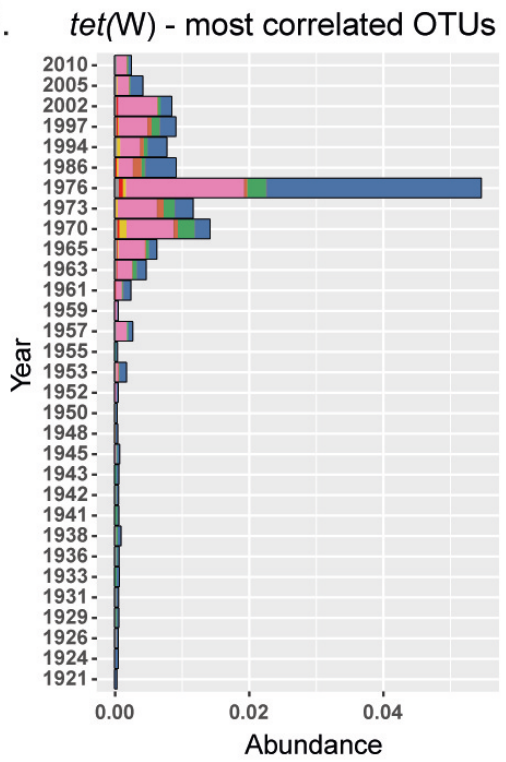

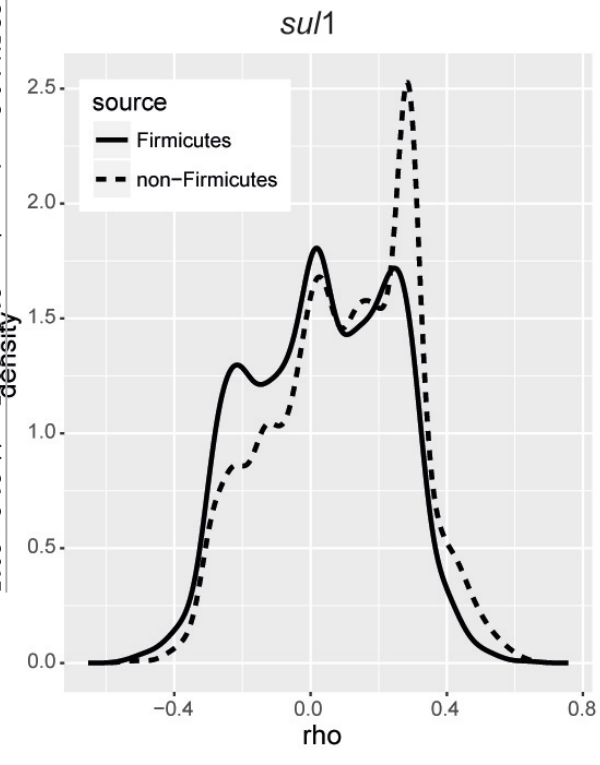

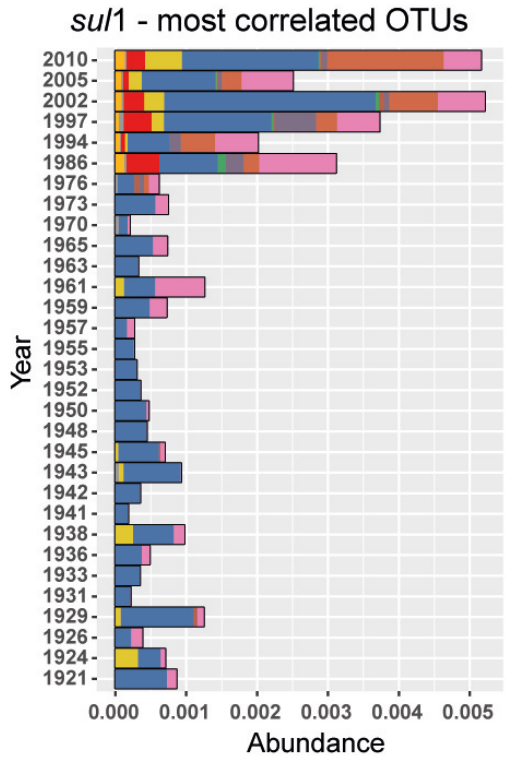

OTU

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- Otu01612;*Lachnoclostridium*;Firmicutes
- Otu00262;Clostridiaceae_1_unclassified;Firmicutes
- Otu00528;Clostridiales_unclassified;Firmicutes
- Otu01577;*Ruminococcus*_1;Firmicutes
- Otu00084;Ruminococcaceae_unclassified;Firmicutes
- Otu00908;Ruminococcaceae_unclassified;Firmicutes
- Otu02280;*Epulopiscium*;Firmicutes
- Otu01131;Verrucomicrobiales_unclassified;Verrucomicrobia
- Otu00529;*Geobacillus*;Firmicutes

OTU

- Otu00318;*Mycobacterium*;Actinobacteria
- Otu00382;Caldilineaceae_unclassified;Chloroflexi
- Otu00975;*Ruminiclostridium*_1;Firmicutes
- Otu03004;*Symbiobacterium*;Firmicutes
- Otu03302;Actinobacteria_unclassified;Actinobacteria
- Otu00155;*Hyphomicrobium*;Proteobacteria
- Otu00604;Verrucomicrobia_unclassified;Verrucomicrobia
- Otu00853;Subgroup_6_unclassified;Acidobacteria
- Otu02777;*Tessaracoccus*;Actinobacteria
- Otu01652;Planctomycetaceae_unclassified;Planctomycetes