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.

Tetracycline binds to the elongating ribosome, affecting translation, and therefore resistance can be acquired through diverse mechanisms (*Davies & Davies, 2010*; *Roberts & Schwarz, 2016*). *tet*(W) is one of a series of ARGs conferring 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 & Schwarz, 2016*). Our analysis suggest that medical historical use (1995–1970) fits well with the observed peak of relative accumulation of *tet*(W) in the seed bank DNA, which was 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, McFall-Ngai & Relman, 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 performance of anaerobic digesters used in wastewater treatment has also shown a highly significant increase in the relative abundance of spore-forming *Firmicutes* after treatment with a concentration of 20 mg/L of tetracycline (*Xiong, Harb & Hong, 2017*). Overall the data suggest that antibiotics such as tetracycline might select for specific groups of *Firmicutes* that can be later found in the seed bank archives.

The same analysis performed on sulfonamides, another class of antibiotics with an industrial history, shows a different trend. Sulfonamide drugs were also among the earliest antibiotics discovered. The legacy of mass production of sulfonamides is reflected in one of the most broadly disseminated cases of drug resistance, both in terms of prevalence and taxonomy (*Aminov, 2010*). Resistance to this class of antibiotic is almost universally associated to genetic mobile elements that confer a fitness advantage to the recipient bacteria as shown in the case of non-pathogenic *Escherichia coli* (*Enne et al., 2004*). The abundance of *sul*1 may thus be indicative of a dissemination trend of certain widespread mobile genetic elements (e.g., class-1 integrons) (*Gillings, 2014*; *Skold, 1976*; *Skold, 2000*) that may well carry other resistance elements. Horizontal gene transfer mediated by mobile genetic elements is considered a major pathway of ARG dissemination (*Bengtsson-Palme, Kristiansson & Larsson, 2017*; *Berglund, 2015*). This particular mechanism of ARG dissemination overcomes taxonomic barriers, probably explaining the wide taxonomic spectrum of bacterial seed bank groups correlated to *sul*1 quantification in the sediments.

The quantification of *sul*1 in the sedimentary record in the 1970s matches early prescription history of this antibiotic class (Table S1). More recent detection could

be correlated to changes in guidelines to reduce usage of penicillin derivatives (such as co-amoxicillin) for uncomplicated urinary tract infection in favor of cotrimoxazole (Sulfamethoxazol-Trimethoprim combination), which may partially explain the common occurrence of *sul*1 resistance gene in the seed bank DNA especially after 2005 (Table S1). At this time medical guidelines changed given the 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 (4,581 strains), which has prompted clinicians to use sulfonamides instead.

CONCLUSIONS

Previous studies of 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 environment (*Graham et al., 2016*; *Knapp et al., 2010*; *Thevenon et al., 2012*), and suggest that reducing non-therapeutic antibiotic use may 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 accumulation in soil 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 results generate valuable information for the debate regarding the long-term effect of the antibiotic era as we show that antibiotics 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 and persistent cellular structures and their potential for further spreading of ARGs in the environment. Importantly however, we hereby 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.

ADDITIONAL INFORMATION AND DECLARATIONS

Funding

The Swiss National Science Foundation (Interdisciplinary grant CR23I2_162810) provided funding for Sevasti Filippidou and Thomas Junier. Helmut Bürgmann acknowledges support by the Swiss National Science Foundation (NFP72 grant 407240_167116). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Grant Disclosures

The following grant information was disclosed by the authors:
The Swiss National Science Foundation: Interdisciplinary grant CR23I2_162810, NFP72 grant 407240_167116.

Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Laura Madueño, Zhanna Bayrychenko and Karin Beck performed the experiments, contributed reagents/materials/analysis tools, reviewed drafts of the paper.
- Christophe Paul conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.
- Thomas Junier performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.
- Sevasti Filippidou performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, reviewed drafts of the paper.
- Gilbert Greub conceived and designed the experiments, contributed reagents/materials/analysis tools, wrote the paper, reviewed drafts of the paper, historical data.
- Helmut Bürgmann and Pilar Junier conceived and designed the experiments, contributed reagents/materials/analysis tools, wrote the paper, reviewed drafts of the paper.

DNA Deposition

The following information was supplied regarding the deposition of DNA sequences:
GenBank: PRJNA396276.

Data Availability

The following information was supplied regarding data availability:
The raw data has been provided as a Supplemental File.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.4197#supplemental-information.

REFERENCES

Abecasis AB, Serrano M, Alves R, Quintais L, Pereira-Leal JB, Henriques AO. 2013. A genomic signature and the identification of new sporulation genes. *Journal of Bacteriology* **195**:2101–2115 DOI 10.1128/JB.02110-12.

Aminov RI. 2009. The role of antibiotics and antibiotic resistance in nature. *Environmental Microbiology* **11**:2970–2988 DOI 10.1111/j.1462-2920.2009.01972.x.

Aminov RI. 2010. A brief history of the antibiotic era: lessons learned and challenges for the future. *Frontiers in Microbiology* **1**:Article 134 DOI 10.3389/fmicb.2010.00134.

Aminov RI, Mackie RI. 2007. Evolution and ecology of antibiotic resistance genes. *FEMS Microbiology Letters* **271**:147–161 DOI 10.1111/j.1574-6968.2007.00757.x.

Baquero F, Martinez JL, Canton R. 2008. Antibiotics and antibiotic resistance in water environments. *Current Opinion in Biotechnology* **19**:260–265 DOI 10.1016/j.copbio.2008.05.006.

Bengtsson-Palme J, Kristiansson E, Larsson DGJ. 2017. Environmental factors influencing the development and spread of antibiotic resistance. *FEMS Microbiology Reviews* Epub ahead of print Oct 24 2017 DOI 10.1093/femsre/fux053.

Berglund B. 2015. Environmental dissemination of antibiotic resistance genes and correlation to anthropogenic contamination with antibiotics. *Infection Ecology & Epidemiology* **5**:Article 28564 DOI 10.3402/iee.v5.28564.

Bhullar K, Waglechner N, Pawlowski A, Koteva K, Banks ED, Johnston MD, Barton HA, Wright GD. 2012. Antibiotic resistance is prevalent in an isolated cave microbiome. *PLOS ONE* **7**:e34953 DOI 10.1371/journal.pone.0034953.

Blair JM, Webber MA, Baylay AJ, Ogbolu DO, Piddock LJ. 2015. Molecular mechanisms of antibiotic resistance. *Nature Reviews Microbiology* **13**:42–51 DOI 10.1038/nrmicro3380.

Bonvin F, Rutler R, Chevre N, Halder J, Kohn T. 2011. Spatial and temporal presence of a wastewater-derived micropollutant plume in Lake Geneva. *Environmental Science & Technology* **45**:4702–4709 DOI 10.1021/es2003588.

Browne HP, Forster SC, Anonye BO, Kumar N, Neville BA, Stares MD, Goulding D, Lawley TD. 2016. Culturing of 'unculturable' human microbiota reveals novel taxa and extensive sporulation. *Nature* **533**:543–546 DOI 10.1038/nature17645.

Cann I, Bernardi RC, Mackie RI. 2016. Cellulose degradation in the human gut: ruminococcus champanellensis expands the cellulosome paradigm. *Environmental Microbiology* **18**:307–310 DOI 10.1111/1462-2920.13152.

Carlet J, Collignon P, Goldmann D, Goossens H, Gyssens IC, Harbarth S, Jarlier V, Levy SB, N'Doye B, Pittet D, Richtmann R, Seto WH, Van der Meer JW, Voss A. 2011. Society's failure to protect a precious resource: antibiotics. *Lancet* **378**:369–371 DOI 10.1016/S0140-6736(11)60401-7.

Chassard C, Delmas E, Robert C, Lawson PA, Bernalier-Donadille A. 2012. Ruminococcus champanellensis sp. nov., a cellulose-degrading bacterium from human gut microbiota. *International Journal of Systematic and Evolutionary Microbiology* **62**:138–143 DOI 10.1099/ijs.0.027375-0.

Corella JP, Brauer A, Mangili C, Rull V, Vegas-Vilarrubia T, Morellon M, Valero-Garces BL. 2012. The 1.5-ka varved record of Lake Montcortes (southern Pyrenees, NE Spain). *Quaternary Research* **78**:323–332 DOI 10.1016/j.yqres.2012.06.002.

Crost EH, Tailford LE, Monestier M, Swarbreck D, Henrissat B, Crossman LC, Juge N. 2016. The mucin-degradation strategy of Ruminococcus gnavus: the importance of intramolecular trans-sialidases. *Gut Microbes* **7**:302–312 DOI 10.1080/19490976.2016.1186334.

Czekalski N, Berthold T, Caucci S, Egli A, Bürgmann H. 2012. Increased levels of multiresistant bacteria and resistance genes after wastewater treatment and their

dissemination into lake geneva, Switzerland. *Frontiers in Microbiology* **3**:Article 106 DOI 10.3389/fmicb.2012.00106.

Czekalski N, Gascon Diez E, Bürgmann H. 2014. Wastewater as a point source of antibiotic-resistance genes in the sediment of a freshwater lake. *ISME Journal* **8**:1381–1390 DOI 10.1038/ismej.2014.8.

Davies J, Davies D. 2010. Origins and evolution of antibiotic resistance. *Microbiology and Molecular Biology Reviews* **74**:417–433 DOI 10.1128/MMBR.00016-10.

D'Costa VM, King CE, Kalan L, Morar M, Sung WWL, Schwarz C, Froese D, Zazula G, Calmels F, Debruyne R, Golding GB, Poinar HN, Wright GD. 2011. Antibiotic resistance is ancient. *Nature* **477**:457–461 DOI 10.1038/nature10388.

De Vries LE, Vallès Y, Agersø Y, Vaishampayan PA, García-Montaner A, Kuehl JV, Christensen H, Barlow M, Francino MP. 2011. The gut as reservoir of antibiotic resistance: microbial diversity of tetracycline resistance in mother and infant. *PLOS ONE* **6**:e21644 DOI 10.1371/journal.pone.0021644.

Dethlefsen L, McFall-Ngai M, Relman DA. 2007. An ecological and evolutionary perspective on human-microbe mutualism and disease. *Nature* **449**:811–818 DOI 10.1038/nature06245.

Devarajan N, Laffite A, Graham ND, Meijer M, Prabakar K, Mubedi JI, Elongo V, Mpiana PT, Ibelings BW, Wildi W, Pote J. 2015. Accumulation of clinically relevant antibiotic-resistance genes, bacterial load, and metals in freshwater lake sediments in Central Europe. *Environmental Science and Technology* **49**:6528–6537 DOI 10.1021/acs.est.5b01031.

Driks A. 2002. Overview: development in bacteria: spore formation in Bacillus subtilis. *Cellular and Molecular Life Science* **59**:389–391 DOI 10.1007/s00018-002-8430-x.

Enne VI, Bennett PM, Livermore DM, Hall LM. 2004. Enhancement of host fitness by the sul2-coding plasmid p9123 in the absence of selective pressure. *Journal of Antimicrobial Chemotherapy* **53**:958–963 DOI 10.1093/jac/dkh217.

FOPH. 2016. Swiss antibiotic resistance report 2016. In: Heim HACD, ed. Usage of antibiotics and occurrence of antibiotic resistance in bacteria from humans and animals in Switzerland. Federal Office of Public Health, 176.

Gillings MR. 2014. Integrons: past, present, and future. *Microbiology and Molecular Biology Reviews* **78**:257–277 DOI 10.1128/MMBR.00056-13.

Goh EB, Yim G, Tsui W, McClure J, Surette MG, Davies J. 2002. Transcriptional modulation of bacterial gene expression by subinhibitory concentrations of antibiotics. *Proceedings of the National Academy of Sciences of the United States of America* **99**:17025–17030 DOI 10.1073/pnas.252607699.

Graham DW, Knapp CW, Christensen BT, McCluskey S, Dolfing J. 2016. Appearance of β-lactam resistance genes in agricultural soils and clinical isolates over the 20th century. *Scientific Reports* **6**:21550 DOI 10.1038/srep21550.

Haller L, Tonolla M, Zopfi J, Peduzzi R, Wildi W, Pote J. 2011. Composition of bacterial and archaeal communities in freshwater sediments with different contamination levels (Lake Geneva, Switzerland). *Water Research* **45**:1213–1228 DOI 10.1016/j.watres.2010.11.018.

Herlemann DP, Labrenz M, Jurgens K, Bertilsson S, Waniek JJ, Andersson AF. 2011. Transitions in bacterial communities along the 2000 km salinity gradient of the Baltic Sea. *ISME Journal* **5**:1571–1579 DOI 10.1038/ismej.2011.41.

Hernandez-Eugenio G, Fardeau ML, Cayol JL, Patel BK, Thomas P, Macarie H, Garcia JL, Ollivier B. 2002. Clostridium thiosulfatireducens sp. nov., a proteolytic, thiosulfate- and sulfur-reducing bacterium isolated from an upflow anaerobic sludge blanket (UASB) reactor. *International Journal of Systematic and Evolutionary Microbiology* **52**:1461–1468 DOI 10.1099/00207713-52-5-1461.

Heuer H, Smalla K. 2007. Manure and sulfadiazine synergistically increased bacterial antibiotic resistance in soil over at least two months. *Environmental Microbiology* **9**:657–666 DOI 10.1111/j.1462-2920.2006.01185.x.

Horino H, Fujita T, Tonouchi A. 2014. Description of Anaerobacterium chartisolvens gen. nov., sp. nov., an obligately anaerobic bacterium from Clostridium rRNA cluster III isolated from soil of a Japanese rice field, and reclassification of Bacteroides cellulosolvens Murray, others, 1984 as Pseudobacteroides cellulosolvens gen. nov., comb. nov. *International Journal of Systematic and Evolutionary Microbiology* **64**:1296–1303 DOI 10.1099/ijs.0.059378-0.

Knapp CW, Dolfing J, Ehlert PAI, Graham DW. 2010. Evidence of increasing antibiotic resistance gene abundances in archived soils since 1940. *Environmental Science & Technology* **44**:580–587 DOI 10.1021/es901221x.

Koinig KA, Shotyk W, Lotter AF, Ohlendorf C, Sturm M. 2003. 9000 years of geochemical evolution of lithogenic major and trace elements in the sediment of an alpine lake—the role of climate, vegetation, and land-use history. *Journal of Paleolimnology* **30**:307–320 DOI 10.1023/A:1026080712312.

Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. 2013. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Applied and Environmental Microbiology* **79**:5112–5120 DOI 10.1128/AEM.01043-13.

Kümmerer K. 2009. Antibiotics in the aquatic environment–a review–Part I. *Chemosphere* **75**:417–434 DOI 10.1016/j.chemosphere.2008.11.086.

Lazzarotto J, Klein A. 2012. *Physical-chemical changes in the waters of Lake Geneva (major elements).* Lausanne: Commission internationale pour la protection des eaux du Léman (CIPEL).

Lee ZM, Bussema 3rd C, Schmidt TM. 2009. rrnDB: documenting the number of rRNA and tRNA genes in bacteria and archaea. *Nucleic Acids Research* **37**:D489–D493 DOI 10.1093/nar/gkn689.

Lennon JT, Jones SE. 2011. Microbial seed banks: the ecological and evolutionary implications of dormancy. *Nature Reviews Microbiology* **9**:119–130 DOI 10.1038/nrmicro2504.

Marti E, Variatza E, Balcazar JL. 2014. The role of aquatic ecosystems as reservoirs of antibiotic resistance. *Trends in Microbiology* **22**:36–41 DOI 10.1016/j.tim.2013.11.001.

Martinez JL. 2008. Antibiotics and antibiotic resistance genes in natural environments. *Science* **321**:365–367 DOI 10.1126/science.1159483.

McMurdie PJ, Holmes S. 2013. Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLOS ONE* **8**:e61217 DOI 10.1371/journal.pone.0061217.

Miller DA, Suen G, Bruce D, Copeland A, Cheng JF, Detter C, Goodwin LA, Han CS, Hauser LJ, Land ML, Lapidus A, Lucas S, Meincke L, Pitluck S, Tapia R, Teshima H, Woyke T, Fox BG, Angert ER, Currie CR. 2011. Complete genome sequence of the cellulose-degrading bacterium Cellulosilyticum lentocellum. *Journal of Bacteriology* **193**:2357–2358 DOI 10.1128/JB.00239-11.

Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, McGlinn D, Minchin PR, O'Hara RB, Simpson GL, Solymos P, Henry M, Stevens H, Szoecs E, Wagner H. 2017. Vegan: community ecology package. R package version 2.4-3. *Available at https://CRAN.R-project.org/package=vegan*.

Patel GB, Khan AW, Agnew BJ, Colvin JR. 1980. Isolation and characterization of an anaerobic, cellulolytic microorganism, acetivibrio cellulolyticus gen. nov., sp. nov. †. *International Journal of Systematic and Evolutionary Microbiology* **30**:179–185 DOI 10.1099/00207713-30-1-179.

Pote J, Haller L, Loizeau JL, Garcia Bravo A, Sastre V, Wildi W. 2008. Effects of a sewage treatment plant outlet pipe extension on the distribution of contaminants in the sediments of the Bay of Vidy, Lake Geneva, Switzerland. *Bioresource Technology* **99**:7122–7131 DOI 10.1016/j.biortech.2007.12.075.

Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glockner FO. 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Research* **41**:D590–D596 DOI 10.1093/nar/gks1219.

R Core Team. 2014. R: a language and environment for statistical computing. Vienna: The R Project for Statistica Computing. *Available at http://www.r-project.org*.

Roberts MC, Schwarz S. 2016. Tetracycline and phenicol resistance genes and mechanisms: importance for agriculture, the environment, and humans. *Journal of Environmental Quality* **45**:576–592 DOI 10.2134/jeq2015.04.0207.

Sauvain L, Bueche M, Junier T, Masson M, Wunderlin T, Kohler-Milleret R, Diez EG, Loizeau JL, Tercier-Waeber ML, Junier P. 2014. Bacterial communities in trace metal contaminated lake sediments are dominated by endospore-forming bacteria. *Aquatic Sciences* **76**:S33–S46 DOI 10.1007/s00027-013-0313-8.

Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn DJ, Weber CF. 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and Environmental Microbiology* **75**:7537–7541 DOI 10.1128/AEM.01541-09.

Skold O. 1976. R-factor-mediated resistance to sulfonamides by a plasmid-borne, drug-resistant dihydropteroate synthase. *Antimicrobial Agents and Chemotherapy* **9**:49–54 DOI 10.1128/AAC.9.1.49.

Skold O. 2000. Sulfonamide resistance: mechanisms and trends. *Drug Resistance Updates* **3**:155–160 DOI 10.1054/drup.2000.0146.

Taylor NG, Verner-Jeffreys DW, Baker-Austin C. 2011. Aquatic systems: maintaining, mixing and mobilising antimicrobial resistance? *Trends in Ecology & Evolution* **26**:278–284 DOI 10.1016/j.tree.2011.03.004.

Thevenon F, Adatte T, Wildi W, Pote J. 2012. Antibiotic resistant bacteria/genes dissemination in lacustrine sediments highly increased following cultural eutrophication of Lake Geneva (Switzerland). *Chemosphere* **86**:468–476 DOI 10.1016/j.chemosphere.2011.09.048.

Van Schaik W. 2015. The human gut resistome. *Philosophical Transactions of the Royal Society B: Biological Sciences* **370**:Article 20140087 DOI 10.1098/rstb.2014.0087.

Vuillemin A, Ariztegui D, Leavitt PR, Bunting L, The PASADO Science Team. 2016. Recording of climate and diagenesis through sedimentary DNA and fossil pigments at Laguna Potrok Aike, Argentina. *Biogeosciences* **13**:2475–2492 DOI 10.5194/bg-13-2475-2016.

Walsh F, Ingenfeld A, Zampicolli M, Hilber-Bodmer M, Frey JE, Duffy B. 2011. Real-time PCR methods for quantitative monitoring of streptomycin and tetracycline resistance genes in agricultural ecosystems. *Journal of Microbiological Methods* **86**:150–155 DOI 10.1016/j.mimet.2011.04.011.

Wright GD. 2010. Antibiotic resistance in the environment: a link to the clinic? *Current Opinion in Microbiology* **13**:589–594 DOI 10.1016/j.mib.2010.08.005.

Wunderlin T, Corella J, Junier T, Bueche M, Loizeau J-L, Girardclos SP, Junier P. 2014a. Endospore-forming bacteria as new proxies to assess impact of eutrophication in Lake Geneva (Switzerland,ÄìFrance). *Aquatic Sciences* **76**:103–116 DOI 10.1007/s00027-013-0329-0.

Wunderlin T, Junier T, Paul C, Jeanneret N, Junier P. 2016. Physical isolation of endospores from environmental samples by targeted lysis of vegetative cells. *Journal of Visualized Experiments* **2016(107)**:e53411 DOI 10.3791/53411.

Wunderlin T, Junier T, Roussel-Delif L, Jeanneret N, Junier P. 2013. Stage 0 sporulation gene A as a molecular marker to study diversity of endospore-forming *Firmicutes*. *Environmental Microbiology Reports* **5**:911–924 DOI 10.1111/1758-2229.12094.

Wunderlin T, Junier T, Roussel-Delif L, Jeanneret N, Junier P. 2014b. Endospore-enriched sequencing approach reveals unprecedented diversity of *Firmicutes* in sediments. *Environmental Microbiology Reports* **6**:631–639 DOI 10.1111/1758-2229.12179.

Xiong Y, Harb M, Hong PY. 2017. Performance and microbial community variations of anaerobic digesters under increasing tetracycline concentrations. *Applied Microbiology and Biotechnology* **101**:5505–5517 DOI 10.1007/s00253-017-8253-1.

Zhilina TN, Kevbrin VV, Turova TP, Lysenko AM, Kostrikina NA, Zavarzin GA. 2005. Clostridium alkalicellum sp. nov. an obligately alkaliphilic cellulolytic bacterium from a soda lake in the Baikal region. *Mikrobiologiia* **74**:642–653.

6 Dissemination of antibiotic resistance genes associated with the sporobiota in sediments impacted by wastewater

Christophe Paul*, Zhanna Bayrychenko*, Thomas Junier, Sevasti Filippidou, Karin Beck, Matthieu Bueche, Gilbert Greub, Helmut Bürgmann, Pilar Junier

2018, PeerJ (Vol. 6, e4989), DOI: 10.7717/peerj.4989

* These authors contributed equally to this work

Foreword

Wastewater treatment plants are known to be a main source of multiple contaminants in the environments, among them antibiotics and antibiotic-resistant organisms. In many cases, WWTP not only fail to eliminate ARG, they increase their relative abundance. WWTP contribute to ARG dissemination, possibly due to the mixing and high abundance of organisms from multiple origins, the pool of ARG present in the different compartments of the WWTP, and the conditions favoring gene transfer. Due to their ability to withstand harsh conditions, spore-formers are likely to resist wastewater treatment. And due to their prevalence in the human microbiome, they are likely to be found in high abundance in WWTP inflows. Although spores had been suggested to have a significant role in ARG dissemination, no study investigated the (distribution and) dispersal rate of ARG associated to spores.

In this chapter we propose to investigate the role of the release ARG and ARG-bearing organisms in the dissemination of antibiotic resistance through wastewater discharge.

As for the preceding article, this publication was also a collective effort led by collaborators from our laboratory and other institutions. My personal contribution included the analysis of the sequencing data and the bacterial community, part of the statistical analyses, the integration and interpretation of the results, the writing of the manuscript, and the co-supervision of the master student (Zhanna Bayrychenko) who proceeded to the DNA extractions and collaborated on the quantification of ARG.

PeerJ

Dissemination of antibiotic resistance genes associated with the sporobiota in sediments impacted by wastewater

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

[1] Institute of Biology, Laboratory of Microbiology, University of Neuchatel, Neuchâtel, NE, Switzerland
[2] Vital-IT, Swiss Institute of Bioinformatics, Lausanne, Switzerland
[3] Eawag, Swiss Federal Institute of Aquatic Science and Technology, Kastanienbaum, Switzerland
[4] Institute of Microbiology, University Hospital Center, University of Lausanne, Lausanne, Switzerland
[*] These authors contributed equally to this work.

ABSTRACT

Aquatic ecosystems serve as a dissemination pathway and a reservoir of both antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARG). In this study, we investigate the role of the bacterial sporobiota to act as a vector for ARG dispersal in aquatic ecosystems. The sporobiota was operationally defined as the resilient fraction of the bacterial community withstanding a harsh extraction treatment eliminating the easily lysed fraction of the total bacterial community. The sporobiota has been identified as a critical component of the human microbiome, and therefore potentially a key element in the dissemination of ARG in human-impacted environments. A region of Lake Geneva in which the accumulation of ARG in the sediments has been previously linked to the deposition of treated wastewater was selected to investigate the dissemination of *tet*(W) and *sul*1, two genes conferring resistance to tetracycline and sulfonamide, respectively. Analysis of the abundance of these ARG within the sporobiome (collection of genes of the sporobiota) and correlation with community composition and environmental parameters demonstrated that ARG can spread across the environment with the sporobiota being the dispersal vector. A highly abundant OTU affiliated with the genus *Clostridium* was identified as a potential specific vector for the dissemination of *tet*(W), due to a strong correlation with *tet*(W) frequency (ARG copy numbers/ng DNA). The high dispersal rate, long-term survival, and potential reactivation of the sporobiota constitute a serious concern in terms of dissemination and persistence of ARG in the environment.

Submitted 2 March 2018
Accepted 27 May 2018
Published 20 June 2018

Corresponding author
Pilar Junier, pilar.junier@unine.ch

Academic editor
Lesley Hoyles

Additional Information and Declarations can be found on page 17

DOI 10.7717/peerj.4989

Subjects Microbiology, Public Health, Ecotoxicology, Freshwater Biology, Environmental Impacts
Keywords WWTP, ARG, Seed bank, *tet*(W), *sul*1, Dispersal

INTRODUCTION

The prevalence and spread of antibiotic resistance is a pressing global public health issue (*Marti, Variatza & Balcazar, 2014*; *Perry, Westman & Wright, 2014*; *O'Neill, 2015*). Although resistance to antibiotics was traditionally viewed as a clinical problem, attention

How to cite this article Paul et al. (2018), Dissemination of antibiotic resistance genes associated with the sporobiota in sediments impacted by wastewater. **PeerJ 6:e4989; DOI 10.7717/peerj.4989**

has recently been directed at understanding the ecological and environmental processes involved in the dissemination of antibiotic-resistant bacteria (ARB) and their associated resistance genes (ARG) (*Berglund, 2015*; *Bengtsson-Palme, Kristiansson & Larsson, 2017*). The dispersal potential of organisms (or genetic elements) carrying ARG is an important element to understand dissemination of antibiotic resistance in the environment. In terms of dissemination, the so-called "sporobiota" is a portion of the bacterial community of great interest. The sporobiota has been defined as a fraction of the microbial community that can exist in the form of highly transmissible spores, which are spread in the environment and are implicated in host-to-host transmission (*Tetz & Tetz, 2017*). This fraction of the community, which can be considered as part of the seed bank (*Lennon & Jones, 2011*; *Shoemaker & Lennon, 2018*), is prone to high dispersal rates due its non-active physiological state, bypassing limitations for local adaptation (*Bartholomew & Paik, 1966*; *Hubert, 2009*; *Lennon & Jones, 2011*). The sporobiota appears to play a significant role as part of the natural microbiota of humans (*Browne et al., 2016*), and therefore, might be highly relevant for ARG dissemination in human-impacted environments. Moreover, one of the features that has been ascribed to the sporobiome (collection of genes of the sporobiota community) is its potential implication in the spread of antibiotic resistance (*Tetz & Tetz, 2017*; *Bengtsson-Palme, Kristiansson & Larsson, 2017*). Although the initial definition of the sporobiota in humans makes reference to a particular type of spores produced by the phylum Firmicutes (heat-resistant endospores; *Tetz & Tetz, 2017*), it is likely that other spore-like structures are found as part of the environmental sporobiota. Therefore, in this study we use a more comprehensive operational definition based on the properties of the sporobiota. The operational definition used here consists of cellular structures withstanding a harsh extraction method designed originally to enrich in endospores from environmental samples (*Wunderlin et al., 2014*).

Aquatic ecosystems can be considered as priority areas when investigating dispersal of ARG in the environment. On the one hand, aquatic ecosystems have a reservoir function, allowing the mixing of environmental organisms and human/animal pathogens, potentially promoting gene transfer, mainly in sediments and biofilms (*Van Elsas & Bailey, 2002*; *Kenzaka, Tani & Nasu, 2010*; *Drudge, 2012*). On the other hand, the use of water bodies for irrigation, recreational and domestic purposes, and as a drinking water supply (after treatment), constitutes a potential pathway enabling transmission of ARB (or ARG) between hosts through the environment (*Baquero, Martínez & Cantón, 2008*; *Taylor, Verner-Jeffreys & Baker-Austin, 2011*). In particular, water bodies connected to wastewater treatment plants (WWTPs) are a major concern (see *Baquero, Martínez & Cantón, 2008*; *Marti, Variatza & Balcazar, 2014* for reviews in the topic). Many WWTPs not only collect wastewater from domestic sources, but also from hospitals or the food-industry, where antibiotics are extensively used (*Szczepanowski et al., 2009*; *Bouki, Venieri & Diamadopoulos, 2013*; *Rodriguez-Mozaz et al., 2015*; *Xu et al., 2015*). Despite elaborate treatment and disinfection phases put in place in modern WWTPs, ARB and ARG have been shown to be only partially eliminated, thus potentially having an important impact on ARG transfer and dispersal (*LaPara et al., 2011*; *Munir, Wong & Xagoraraki, 2011*; *Dodd, 2012*; *Rizzo et al., 2013*).

Lake Geneva is the largest freshwater lake in Western Europe and constitutes a major source of drinking water. The lake receives wastewater from the surrounding cities. The largest WWTP is located near the city of Lausanne and receives wastewater from both domestic and industrial/clinical sources. Treated wastewater is released into a bay of the lake, the Vidy Bay. Overflow associated with strong rain episodes also contributed to the occasional release of untreated wastewater. Although a natural background of resistance genes is ubiquitous, and in addition the entire Lake Geneva basin is likely affected by human activity, sediments of the Vidy Bay are more significantly impacted in terms of organic and inorganic pollution by the WWTP discharge, due to their immediate proximity to the outlet. Contamination by fecal-indicator bacteria, trace metals, nitrogen, phosphorus, and ARG has been reported (*Haller et al., 2011*; *Czekalski et al., 2012*; *Thevenon et al., 2012*; *Czekalski, Gascón Díez & Bürgmann, 2014*; *Devarajan et al., 2015*). Moreover, ARG and resistant bacteria have been detected close to the drinking water pump of Lausanne, 3.2 km away from the WWTP outflow, highlighting the potential risk for human health from the transfer of ARB and ARG from the environment back into humans (*Czekalski et al., 2012*). Therefore, Lake Geneva constitutes an ideal system to evaluate the role of the sporobiota in ARG dissemination. We have previously shown that the sporobiome of lake sediment preserves a historical record of resistance prevalence using as proxies for ARG the *tet*(W) and *sul*1 genes (*Madueño et al., 2018*) and the same genes were also selected for this study. Although analyzing only two ARG limits the scope of our work and results should therefore not be generalized, currently the low DNA yield resulting from the separation of the sporobiota limits the number of genes that can be assessed simultaneously without total DNA amplification. The *tet*(W) gene confers resistance to tetracycline, a class of natural antibiotics isolated from *Streptomyces* that inhibit protein synthesis (*Roberts, 1996*). The *sul*1 gene confers resistance to sulfonamide, a class of synthetic antibiotics that inhibit the enzyme dihydropteroate synthase (DHPS) in the folic acid pathway (*Sköld, 2001*). Moreover, the *sul*1 gene has been suggested as a useful proxy for monitoring ARG in the environment (*Berendonk et al., 2015*). Both genes are known to be abundant in wastewater-impacted sediments (*Czekalski, Gascón Díez & Bürgmann, 2014*; *Na et al., 2014*; *Rodriguez-Mozaz et al., 2015*), and in particular, the high abundance of these two genes among total bacterial communities was previously reported in sediments from the same location (*Czekalski, Gascón Díez & Bürgmann, 2014*). Sulfonamides and tetracyclines are still in use in human medicine and are the antibiotic classes with the highest and third highest sales numbers, respectively, for the veterinary sector in Switzerland (*Federal Office of Public Health, 2016*).

The aims of the present study were to investigate the accumulation of ARG *tet*(W) and *sul*1 in DNA extracted from sediments treated to enrich the sporobiota and that are impacted differentially by a WWTP discharge. In addition, we analyzed the sporobiota community composition and its relationship to ARG levels, spatial distribution, and characteristics of sediments.

MATERIAL AND METHODS

Site description and sampling

Lake Geneva is the largest freshwater lake of Western Europe, with a volume of 89 km^3, a surface area of 580 km^2 and a maximum depth of 309 m. Vidy Bay is enclosed by the shoreline near Lausanne between St.-Sulpice and Lausanne-Ouchy. The outlet pipe of the WWTP is located 700 m offshore at 35 m depth (46.51197121 N; 6.587423025 E).

The ten cores used for this study were retrieved using standard corers (50 cm length Plexiglas tubes of 6 cm diameter) between July 2011 and May 2012 in three sampling zones differently impacted by the WWTP (*Bueche, 2014*; *Sauvain et al., 2014*): 5–40 m from the outlet pipe of the WWTP ("near", N1–N4); 134–429 m from the outlet pipe ("middle", M1–M4); and 611–956 m from the outlet pipe ("distal", D1–D2). Two sediment layers were analyzed for the cores with the code numbers 1 and 2 (D1, D2, M1, M2, N1, N2) [20]: 0–3 cm and 3–9 cm depth. Three sediment layers were analyzed for the cores with the code numbers 3–4 (M3, M4, N3, N4): 0–1.5 cm, 1.5–3 cm and 3–9 cm depth.

DNA extraction

Sporobiome DNA was obtained using an indirect three step extraction method: (i) extraction of cells from the sediment particles, (ii) separation of spores from the vegetative cells, (iii) DNA extraction from the spores (*Wunderlin et al., 2014*).

The treatment to separate spores from vegetative cells was performed on the biomass from 1.5 grams of sediment collected on one nitrocellulose filter (Merck Millipore, Darmstadt, Germany) per sample, as previously described (*Wunderlin et al., 2014*). The first step consisted of the lysis of vegetative cells by heat, enzymatic agents (lysozyme) and chemicals (Tris-EDTA, NaOH, SDS). The second step consisted of a DNase digestion in order to destroy the free DNA. Treated filters were stored at −20 °C until DNA was extracted from the pre-treated filters using a protocol based on the FastDNA® SPIN kit for soil (MP Biomedicals, Santa Ana, CA, USA) (*Wunderlin et al., 2013*) with the following additional modifications: samples in lysing matrix were submitted to two successive bead-beating steps. Supernatant retrieved from each bead-beating was treated separately according to manufacturer's instructions and DNA extracts were pooled together at the end of the procedure. Pooled DNA was precipitated with 0.3 M Na-acetate and ethanol (99%), stored at −20 °C overnight and centrifuged for 1 h at 21460xg and 4 °C. Supernatant was removed and the pellet was washed with 1 ml of 70% ethanol and centrifuged for 30 min at 21460xg and 4 °C. Supernatant was removed and the residual ethanol was allowed to evaporate at room temperature. Pellet 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).

Real-time quantitative PCR on *tet(W)* and *sul*1 genes

Real-time Taqman®-PCR on *sul*1 and *tet*(W) genes was performed in 384-well plates using a LightCycler®480 Instrument II (Roche, Basel, Switzerland). For *sul*1, the primers used were qSUL653f (5′-CCGTTGGCCTTCCTGTAAAG-3′) and qSUL719r

(5′-TTGCCGATCGCGTGAAGT-3′) with tpSUL1 (*FAM*-CAGCGAGCCTTGCGGCGG-*TAMRA*) probe (*Heuer & Smalla, 2007*). The reaction mix for *sul*1 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 × TaqMan® Fast Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Total reaction volume of 10 µL was reached with PCR-grade water. For *tet* (W), the primers used were tetW_f (5′-CGGCAGCGCAAAGAGAAC-3′) and tetW_r (5-CGGGTCAGTATCCGCAAGTT-3′) with tetW_s (*FAM*-CTGGACGCTCTTACG-*TAMRA*) 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 × TaqMan® Fast Universal PCR Master Mix (Applied Biosystems, Foster City, CA, 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 5×10^7 to 50 gene copies. For *sul*1, control plasmids and standard curves were prepared as previously described (*Heuer & 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 *sul*1 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 gene amplicons 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 performed using Mothur (*Schloss et al., 2009*) following the MiSeq SOP (*Kozich et al., 2013*). The SILVA NR v123 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 1818238 amplicons were obtained (408758 unique sequences). Singletons were removed prior to the clustering into OTUs and corresponded to 373374 sequences. Average neighbor clustering of the 1444864 remaining sequences (35384 unique sequences) with an identity threshold of 97% led to the identification of 6390 OTUs. Sequencing data was deposited to NCBI under the Bioproject accession number PRJNA396277.

Statistical and multivariate analysis

Community and statistical analyses were performed using R version 3.4.0 (*R Core Team, 2014*) and the *phyloseq* and *vegan* packages (*McMurdie & Holmes, 2013*; *Oksanen et al.,*

2017). The distance of samples to the WWTP outflow is a combination (euclidian distance) of the distance between the sample and the pipe on the east axis, the north axis (based on CH coordinates) and the water column depth. It is expressed in meters. The difference in ARG levels between the zones (near, middle and distal) was tested using analysis of variance (ANOVA) and Tukey's test for pairwise comparisons. Pairwise correlations between environmental parameters (organic carbon, total nitrogen, and concentration of cadmium, copper, iron, manganese, aluminum, zinc and arsenic) and ARG abundance/frequency were calculated using Spearman's rank correlation coefficient. This correlation coefficient was selected because we did not assume a normal distribution of the variables. The organic carbon, total nitrogen, and metal concentrations were obtained from previous studies (*Bueche, 2014*; *Sauvain et al., 2014*). *P*-values were adjusted for family-wise error rate using the Holm method. Spearman's rank correlation coefficient was used for calculating the correlations between ARG frequency and the relative abundance of OTUs. To provide a visual representation of the distribution of the correlation coefficient of OTUs with ARG concentration, Kernel density curves were computed from Spearman's correlation coefficients calculated between each ARG and OTU and can be thought of as continuous histograms representing the frequency (density) of the correlation coefficients. The significance of the Spearman's correlation coefficients was tested by correcting the *p*-values using the false discovery rate correction (Benjamini–Hochberg method). This method was selected because of the large number of comparisons performed. Principal component analysis (PCA) was computed on the environmental parameters and ARG abundance/frequency, after standardization (zero mean and unit variance). The environmental parameters included were C_{org}, N_{tot}, metals (listed above), distance to the outlet pipe (Dist_pipe), DNA abundance in ng per gram of sediment (DNA_ng_gSed), and ARG abundance and frequency. Sporobiota community was analyzed by principal coordinates analysis (PCoA), based on Bray-Curtis dissimilarity and Hellinger transformation of the OTU table. Environmental parameters and ARG abundance/frequency were standardized and passively fitted to the ordination. Only significant parameters were displayed ($p < 0.05$).

RESULTS

Prevalence and spatial distribution of *tet*(W) and *sul*1 in sporobiome DNA

To determine the prevalence of the two selected ARG in DNA extracted from the sporobiota and to describe their spatial distribution in sediments impacted by a WWTP, ARG quantification was carried out for three zones located at 5-40 m (near) 134-429 m (middle) and 611–956 m (distal) from the outlet pipe (Fig. 1). These zones were previously shown to be differentially impacted by the release of wastewater, according to the concentration of particulate trace and heavy metals in the sediments and to the bacterial community composition (*Sauvain et al., 2014*). The results of ARG quantification were expressed in two different units. The first unit, ARG abundance, refers to the number of copies of ARG per g of sediment. This reflects accumulation in sediment and represents the total ARG

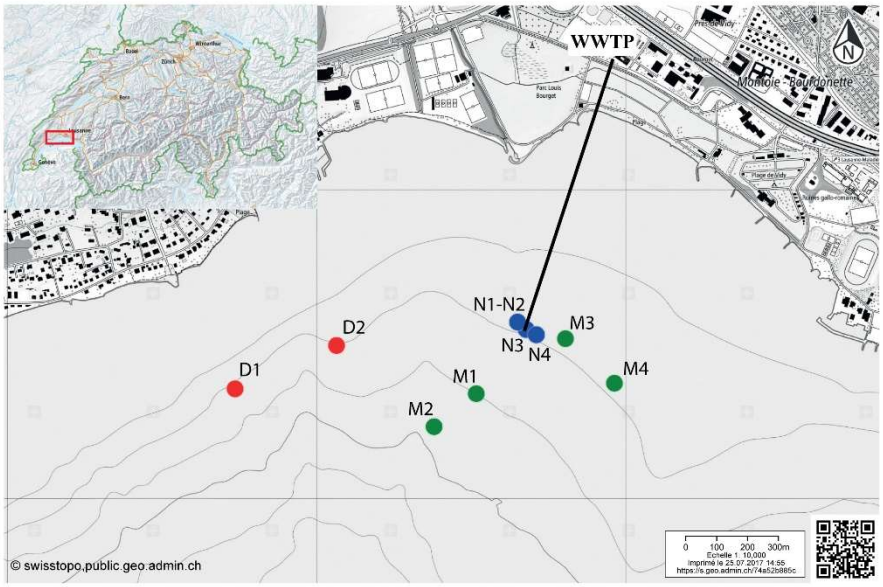


Figure 1 Map indicating the sampling locations in the Vidy Bay, Lake Geneva, Switzerland. Samples were retrieved from three zones differently impacted by the wastewater treatment plant (WWTP). Red circles are for the distal zone (D; 611–956 m from the outlet pipe), green circles are for the middle zone (M; 134–429 m), and blue circle are for the near zone (N; 5–40 m). Outlet pipe of the WWTP is represented by the black line. The original maps were obtained from http://www.swisstopo.ch, owners of the copyright of the image.

Full-size DOI: 10.7717/peerj.4989/fig-1

pool for a given ARG. However, differential microbial load in sediments could bias this measure. Therefore, we used a second unit, ARG frequency, which is defined as the number of copies of ARG per ng of extracted DNA. This allowed us to estimate the enrichment of specific ARG in DNA from the enriched sporobiota. We caution that both approaches are potentially affected by changes in the proportion of different bacterial groups that may not be causally related to either inputs or growth of ARB or local selection of ARG. For example, an increase in abundance of a population without an ARG due to nutrient inputs will result in a reduction of ARG frequency.

Both ARG, *tet*(W) and *sul*1, were detected in DNA at all sampling locations. In all samples, abundance and frequency were higher for *tet*(W) than for *sul* 1 (Table 1). The influence of the WWTP as the main source of sporobiota-associated ARG can be observed in the spatial distribution of ARG abundance. Abundance was highest in samples from the proximal zone and decreased with distance to the outlet pipe (Fig. 2, Figs. S1 and S2). This pattern was confirmed by principal component analysis (PCA; Fig. S1) and Spearman correlation tests (Fig. S3), which showed that ARG abundance was mostly correlated with environmental variables associated with deposition of treated wastewater, such as organic carbon content (C_{org}) and total nitrogen (N_{tot}) in sediments. However, unexpectedly, the

Table 1 Abundance and frequency of antibiotic resistance genes in sediments. Abundance (copies/g of sediment) and frequency (copies/ng of DNA) of two antibiotic resistance genes (*tet*(W) and *sul*1) in the sporobiota, in sediments samples from the Vidy Bay (Lake Geneva, CH).

Sample	Sediment depth	Dist. to the pipe	DNA	*tet*(W)		*sul*1	
	(cm)	(m)	(ng/g sed)	(copies/ng DNA)	(copies/g sed)	(copies/ng DNA)	(copies/g sed)
D1_low	3–9	955.89	3.081	3.01E+03	9.26E+03	4.65E+01	1.43E+02
D1_up	0–3	955.89	5.385	2.81E+03	1.51E+04	9.33E+01	5.02E+02
D2_low	3–9	610.99	2.461	3.73E+03	9.19E+03	5.17E+01	1.27E+02
D2_up	0–3	610.99	3.805	3.27E+03	1.24E+04	8.58E+01	3.26E+02
M1_low	3–9	259.15	3.066	2.56E+04	7.85E+04	2.29E+02	7.02E+02
M1_up	0–3	259.15	3.530	1.19E+04	4.19E+04	3.22E+02	1.14E+03
M2_low	3–9	429.04	2.391	2.50E+04	5.98E+04	1.78E+02	4.25E+02
M2_up	0–3	429.04	2.166	1.19E+04	2.58E+04	3.82E+02	8.26E+02
M3_low	3–9	133.76	0.773	7.37E+03	5.69E+03	2.80E+01	2.17E+01
M3_med	1.5–3	133.76	1.104	9.56E+03	1.06E+04	1.75E+02	1.93E+02
M3_up	0–1.5	133.76	2.963	1.38E+04	4.10E+04	4.97E+02	1.47E+03
M4_low	3–9	335.80	0.968	8.85E+03	8.57E+03	1.65E+02	1.60E+02
M4_med	1.5–3	335.80	1.958	2.43E+03	4.75E+03	6.56E+01	1.28E+02
M4_up	0–1.5	335.80	1.208	8.93E+03	1.08E+04	5.83E+02	7.04E+02
N1_low	3–9	5.39	2.313	7.24E+03	1.67E+04	1.26E+02	2.92E+02
N1_up	0–3	5.39	4.802	1.21E+04	5.79E+04	3.21E+02	1.54E+03
N2_low	3–9	5.39	4.183	1.46E+04	6.12E+04	2.32E+02	9.69E+02
N2_up	0–3	5.39	7.658	1.81E+04	1.38E+05	6.20E+02	4.75E+03
N3_low	3–9	36.14	2.497	1.31E+04	3.27E+04	1.57E+02	3.92E+02
N3_med	1.5–3	36.14	2.428	1.36E+04	3.31E+04	4.08E+02	9.90E+02
N3_up	0–1.5	36.14	2.617	1.29E+04	3.38E+04	4.18E+02	1.09E+03
N4_low	3–9	39.61	13.900	1.14E+04	1.58E+05	7.80E+02	1.08E+04
N4_med	1.5–3	39.61	10.515	1.72E+04	1.81E+05	1.34E+03	1.41E+04
N4_up	0–1.5	39.61	7.115	1.92E+04	1.37E+05	1.60E+03	1.14E+04

R^2 calculated for the linear regression established between ARG abundance and distance to the outlet pipe in our case (Fig. S2) was generally low.

Measures of ARG frequency showed that *tet*(W) and *sul*1 genes had different enrichment patterns in the sporobiota. In the case of *sul*1, ARG frequency decreases in relation to the distance to the outlet pipe (Fig. 2, Fig. S2). A decrease in frequency could be attributed to dilution of a population with high *sul*1 frequency (originating from the wastewater) within the environmental sporobiota community. The frequency of *tet*(W) also decreased in relation to the distance to the outlet pipe, but its pattern of distribution was more complex. The highest *tet*(W) frequencies were found in two middle zone samples (M1_low and M2_low, Fig. 2, Fig. S1). Both correlation tests (Fig. S3) and PCA with the environmental variables (Fig. S1B) indicated a significant correlation between *tet*(W) frequency and certain metals (Fe, As, and Cu).

Sporobiota community composition

Several studies have demonstrated a strong impact of WWTP discharge on bacterial community composition, which is shown by changes in the community in impacted

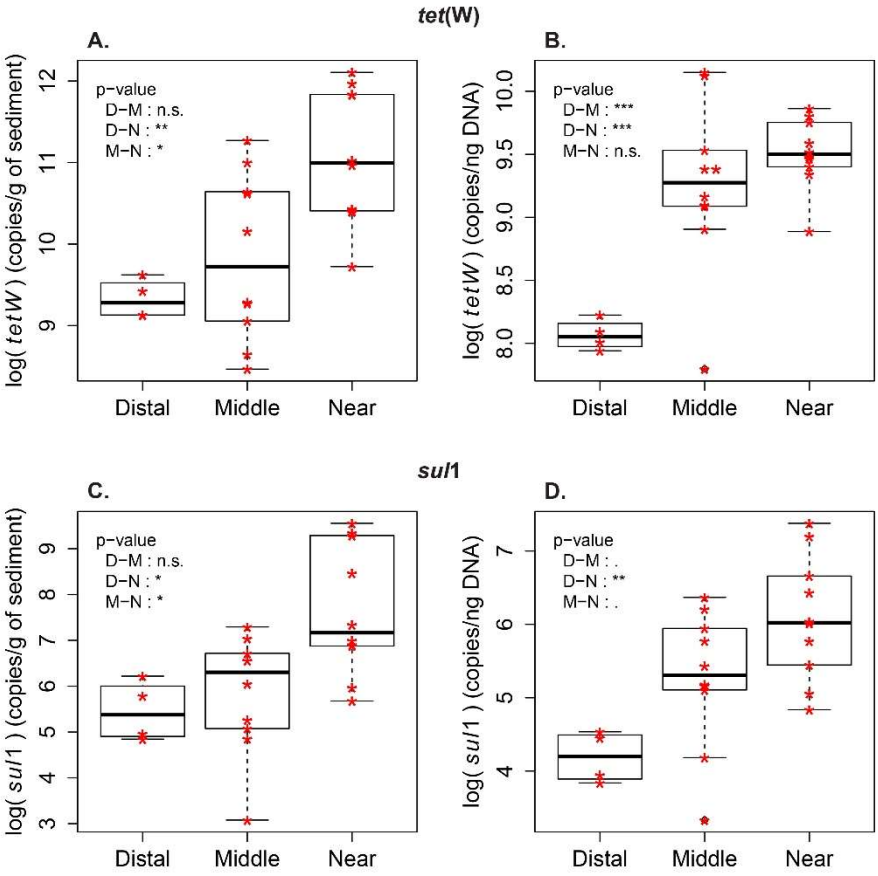


Figure 2 Abundance and frequency of ARG in relationship to distance to the wastewater treatment plant. Boxplots for *tet* (W) and *sul*1 distribution in the samples from distal [D], middle (M) and near (N) sampling zones. (A) *tet* (W) log-transformed gene abundance (copies/g of sediment). (B) *tet* (W) log-transformed gene frequency (copies/ng DNA). (C) *sul*1 log-transformed gene abundance (copies/g of sediment). (D) *sul*1 log-transformed gene frequency (copies/ng DNA). Pairwise comparison with Tuckey's test was performed to compare the ARG level between the zones (Distal-Middle, Distal-Near, Middle-Near). Significance codes of *p*-values: $0 < *** < 0.001 < ** < 0.01 < * < 0.05 < . < 0.1$. (n.s.) stands for "not significant".

Full-size DOI: 10.7717/peerj.4989/fig-2

sediments compared to remote sites (*LaPara et al., 2011*; *Czekalski, Gascón Díez & Bürgmann, 2014*; *Sauvain et al., 2014*). In previous studies using a different sequencing technology (pyrosequencing), the total bacterial communities analyzed from the same sediments were highly divergent, forming at least four distinct groups depending on the distance to the outlet pipe and the concentration of heavy and trace metals in the sediments (*Bueche, 2014*; *Sauvain et al., 2014*). In contrast, the sporobiota community has a low degree of variability among impacted and supposedly less impacted sites (Fig. 3), suggesting that

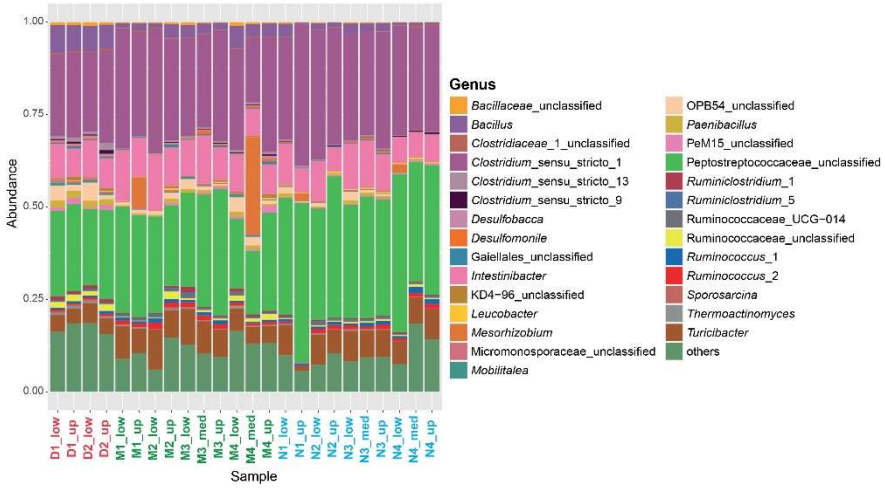


Figure 3 Characterization of the sporobiome community in sediments. Composition of the sporobiome community in sediments from Vidy Bay (Lake Geneva) based on 16S rRNA gene amplicon sequencing. Relative abundance of the most abundant genera (>0.2% of the total community) is represented.

Full-size DOI: 10.7717/peerj.4989/fig-3

the distance to the wastewater outlet pipe had a relatively small impact on sporobiota community composition. Firmicutes are the dominant phylum in all samples (Fig. S4), accounting for at least 85.2% of the sporobiota community, except in sample taken from the middle area at a depth of 1.5–3 cm (M4_med; 65.9%). This is in agreement with a previous study demonstrating an enrichment of 83.9–90.6% in Firmicutes using the same method for the isolation of spores (*Wunderlin et al., 2014*). Among the non-Firmicutes representatives, we found several groups for which the production of spores is so far not reported and that could represent potential contamination with vegetative cells that withstood the extraction procedure (see below). Nevertheless, we performed the analysis with the entire community as our definition of the sporobiota considers the resilience to the extraction method, rather than phylogenetic affiliation.

In terms of relative abundance, 43.1% of the whole community was identical between all samples. The two most abundant genera, *Clostridium*_sensu_stricto_1 and an unclassified Peptococcaceae, were the same for all samples (except M4_med), with an average relative abundance of 57.3%. Both are widely represented in the human gut (*Lozupone et al., 2012*), which is also the case for other subdominant genera, including *Intestinibacter* (9.7%), *Turicibacter* (6.7%), and *Ruminococcus*_2 (0.9%). These findings suggest a common origin of the sporobiome and point again at the WWTP as the main source of the sporobiome community depositing in the lake sediment in the area studied here.

Despite the high homogeneity of the sporobiota community, principal coordinate analysis (PCoA, Fig. 4) reveals a slight variability among the communities. For example, in Axis 1, Bacilli is associated with samples located at a greater distance from the outlet pipe,

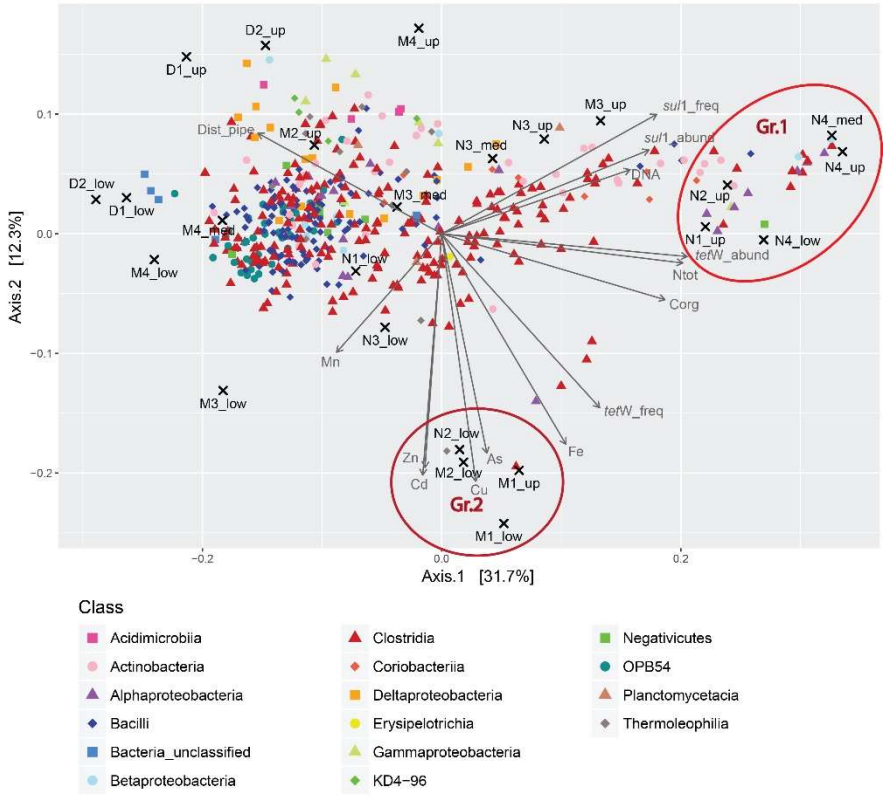


Figure 4 Principal coordinates analysis (PCoA) triplot of the sediment samples based on Bray-Curtis dissimilarity and Hellinger transformation of the OTU table. OTUs represented by less than four sequences in the whole dataset were removed from the analysis. OTUs were classified at genus level (or higher taxonomic rank if not possible). Colors correspond to different orders. Only the 80 most abundant OTUs are shown. Environmental variables (including C_{org}, N_{tot}, trace and heavy metals (TMs), the distance to the outlet pipe (Dist_pipe), DNA abundance in ng/g sediment (DNA_ng_gSed), and ARG abundance (cp_gSed) and frequency (cp_ng)) were standardized and passively fitted to the ordination. Only significant parameters were displayed ($p < 0.05$). Values for C_{org}, N_{tot}, and TMs were obtained from previous studies (*Bueche, 2014*; *Sauvain et al., 2014*) .

Full-size DOI: 10.7717/peerj.4989/fig-4

while different Clostridia are associated to samples closer to the WWTP. While Clostridia are mostly anaerobic and are common in the microbiome of humans and other mammals, Bacilli include numerous aerobic organisms and are commonly found in soils (*Madigan et al., 2015*). Accordingly, Clostridia could be seen as a signature of the human influence in the sporobiota associated with wastewater discharge whereas Bacilli would represent a more environmental sporobiota community.

In addition, PCoA revealed two groups of samples of particular interest (Fig. 4). Group 1 (Gr.1 in Fig. 4; Fig. S5B), which was characterized by high load of C_{org} and N_{tot}, and high abundance/frequency of ARG, was composed of samples from the zone in close

proximity to the WWTP discharge. Among the OTUs associated to this group, the most abundant belong to the genera *Bifidobacteria* and *Collinsella*, two Actinobacteria common in the human microbiome (*Lamendella et al., 2008*; *Thorasin, Hoyles & McCartney, 2015*), albeit unknown for the production of spores. Another abundant genus in this group is *Trichococcus*, a Firmicute found in sewerage infrastructure and WWTP (*Vandewalle et al., 2012*). This group reflects the direct influence of the WWTP (Fig. S5B). In contrast, Group 2 (Gr.2 in Fig. 4; Fig. S5C) clustered samples from both the near and middle zones and was related to elevated concentrations of particulate metals (Zn, Cd, Cu, As, and Fe) and a high frequency of *tet*(W) (Fig. 4). OTUs defining this group belonged mainly to non-Firmicutes (Fig. S5C). Some were affiliated to organisms that have been previously isolated from WWTPs, including Synergistetes (*Wang et al., 2013*), *Syntrophorhabdus* (*Saia et al., 2016*), Anaerolinaceae and *Leptolinae* (*Yamada et al., 2006*). Others were affiliated to uncommon taxa previously isolated from rivers or aquifers, such as Gaiellales (*Albuquerque et al., 2011*) and *Kaistia* (*Jin et al., 2011*). To our knowledge, there is no evidence that these organisms are resistant to high metal contamination.

Correlation tests between OTUs and ARG

To further investigate the relationship between the sporobiota community and ARG distribution, pairwise spearman correlation tests were performed between *tet*(W) frequency and the relative abundance of each OTU. For both *tet*(W) and *sul*1, the results indicated that only a small number of OTUs correlate with the ARG studied here (Figs. 5A–5B). Interestingly, the 10 most positively correlated OTUs were specific to each ARG. Although environmental correlations alone are not sufficient to identify carriers of antibiotic resistance, the results can help to highlight potential groups that merit further investigation. For example, the three most *tet*(W)- positively correlated OTUs (correlation coefficients ranging from 0.84 to 0.71) all belonged to Firmicutes (Table 2). In particular, one of these OTUs (OTU00002; *Clostridium*_sensu_stricto_1) was highly abundant (over 20% of the relative abundance) in all samples (Fig. 5C). The known bacterial species most closely related to this OTU were *Clostridium celatum, Clostridium saudiense,* and *Clostridium disporicum*, all isolated from the feces of mammals, including humans, swine and rats (*Horn, 1987*; *Angelakis et al., 2014*; *Agergaard et al., 2016*). Although in most *Clostridium* spp. studied so far, tetracycline resistance is conferred by the *tet*(M) gene (*Adams et al., 2002*), *tet*(W) has been detected in a medical isolate from *Clostridium difficile* (*Spigaglia, Barbanti & Mastrantonio, 2008*). A similarity search using this gene as a query showed the detection of homologues in various uncultured environmental *Clostridium* spp. (Supplemental Information 2). The other two most correlated OTUs (Otu00045 and Otu00062) both belonged to the genus *Ruminococcus*_1, closely related to *Ruminococcus callidus* and unclassified *Ruminococcus* spp., respectively. All of these have been reported in human and other mammal feces (*Wang, Cao & Cerniglia, 1997*; *Leser et al., 2002*; *Schmidt et al., 2011*). Another OTU of interest was OTU00008, which represented up to 1.75% of the total community (0.87% in average). OTU00008 was closely related to *Ruminococcus bromii*, one of the predominant species in human, swine, and cattle gut (*Moore, Cato & Holdeman, 1972*; *Klieve et al., 2007*). Although the genus *Ruminococcus* was originally considered as

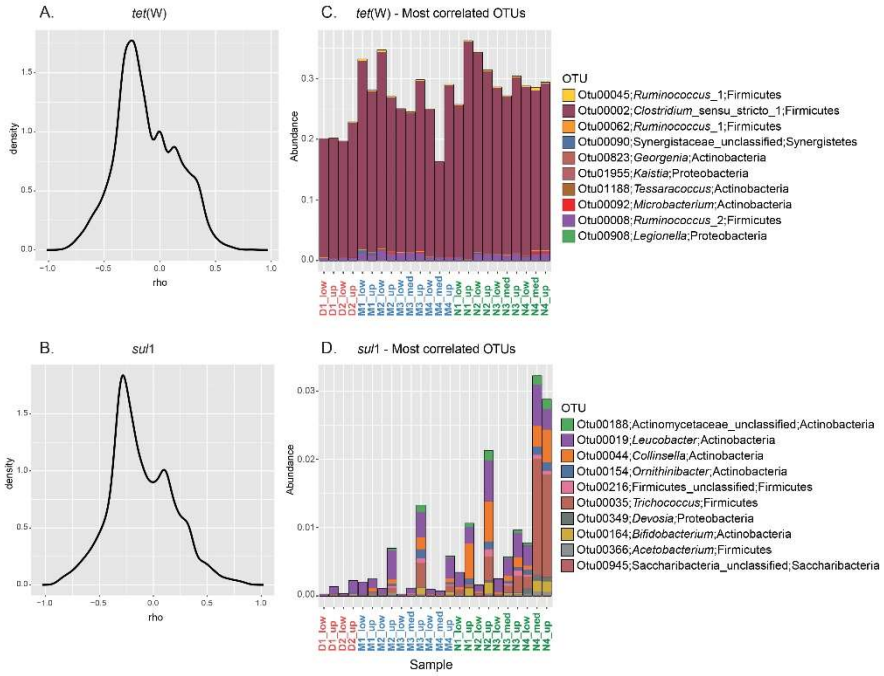


Figure 5 Correlation between specific OTU and frequency of the ARG *tet*(W) and *sul*1. Kernel density curves representing the frequency of the Spearman's correlation coefficients calculated between ARG and OTU and relative abundance of the 10 most positively correlated OTUs for each ARG. (A) Kernel density curves for *tet*(W). (B) Kernel density curves for *sul*1. (C) Relative abundance of OTUs correlated with *tet*(W). (D) Relative abundance of OTUs correlated with *sul*1.

Full-size DOI: 10.7717/peerj.4989/fig-5

to comprise non-spore forming species, a recent report has demonstrated the production of spores by *Ruminococcus* isolated from human colon and rumen (*Mukhopadhya et al., 2018*). This gives additional support to our approach and shows the importance of defining the sporobiota based on its properties, rather than on the reports of spore production in cultured isolates.

In spite of the widespread phylogenetic distribution of *sul*1 (*Aminov, 2010*), the 10 most *sul*1-positively correlated OTUs belonged mainly to Actinobacteria (Table 2). The other most correlated OTUs belonged to Firmicutes, Proteobacteria, and Saccharibacteria (all with a correlation coefficient above 0.8). Unsurprisingly, all these highly correlated OTUs were closely related to organisms commonly reported as part of the human/mammal microbiome and/or are commonly found in WWTPs. Compared to *tet*(W), none of the best correlated OTUs represented a major fraction of the sporobiome community (<0.25% in average, Fig. 5D). However, one has to be careful in the interpretation of these correlations. The system under study is mainly driven by the influence of the WWTP. Most *sul*1-correlated OTUs were related to Group 1 and their abundance correlates with high

Table 2 Correlations between OTU relative abundance and ARG frequency. Each line indicates the correlation coefficient of the 10 most positively correlated OTUs, their associated *p*-values (with and without adjustment for multiple comparison) and the phylogenetic identity of OTUs. Adjusted *p*-value were calculated using the Benjamini–Hochberg method to control the false discovery rate.

OTU	Cor. coeff.	*p*-value	Adj. *p*-value	Phylum	Genus
tet(W)					
Otu00045	0.841	2.58E−07	8.23E−04	Firmicutes	*Ruminococcus_1*
Otu00002	0.805	2.10E−06	1.47E−03	Firmicutes	*Clostridium*_sensu_stricto_1
Otu00062	0.712	9.42E−05	1.06E−02	Firmicutes	*Ruminococcus_1*
Otu00090	0.673	3.09E−04	2.15E−02	Synergistetes	Synergistaceae_unclassified
Otu00823	0.673	3.13E−04	2.15E−02	Actinobacteria	*Georgenia*
Otu01955	0.667	3.66E−04	2.32E−02	Proteobacteria	*Kaistia*
Otu01188	0.663	4.19E−04	2.50E−02	Actinobacteria	*Tessaracoccus*
Otu00092	0.651	5.64E−04	2.91E−02	Actinobacteria	*Microbacterium*
Otu00008	0.638	8.05E−04	3.52E−02	Firmicutes	*Ruminococcus_2*
Otu00908	0.635	8.68E−04	3.62E−02	Proteobacteria	*Legionella*
*sul*1					
Otu00188	0.882	1.18E−08	1.83E−05	Actinobacteria	Actinomycetaceae_unclassified
Otu00019	0.856	9.86E−08	5.66E−05	Actinobacteria	*Leucobacter*
Otu00044	0.850	1.45E−07	5.80E−05	Actinobacteria	*Collinsella*
Otu00154	0.843	2.41E−07	6.70E−05	Actinobacteria	*Ornithinibacter*
Otu00216	0.840	2.86E−07	7.62E−05	Firmicutes	Firmicutes_unclassified
Otu00035	0.835	3.79E−07	8.64E−05	Firmicutes	*Trichococcus*
Otu00349	0.827	6.42E−07	1.17E−04	Proteobacteria	*Devosia*
Otu00164	0.826	6.61E−07	1.17E−04	Actinobacteria	*Bifidobacterium*
Otu00366	0.821	8.69E−07	1.42E−04	Firmicutes	*Acetobacterium*
Otu00945	0.816	1.18E−06	1.76E−04	Saccharibacteria	Saccharibacteria_unclassified

concentrations of C_{org} and N_{tot} and other environmental indicators of the direct influence of treated wastewater release (Fig. S5).

DISCUSSION

Our results show that the effluents from the studied WWTP have a clear impact on the deposition of ARG associated with the bacterial sporobiome in sediments. Higher concentrations of ARG were found in the proximity of the outlet pipe compared to a more distal zone. These results were expected given the reported effect of treated wastewater on the levels of ARG in WWTP-impacted environments. In a study evaluating the dissemination of ARG from a river receiving WWTP discharge and farming runoffs, a decrease of ARG abundance with distance to the source was measured, reflecting a decreasing anthropogenic impact. However, most ARG were detectable even in distant sediments (*Chen et al., 2013*). Another study investigating the spatial distribution of different ARG in relation to WWTP outflow into a lake detected an abundance up to 200 fold higher in the vicinity of the outlet pipe with exponential decline (ARG abundance and concentration) as a function of the distance to the WWTP outlet pipe (*Czekalski, Gascón Díez & Bürgmann, 2014*).

The ARG investigated in the present study appeared to be differently enriched within the sporobiota, which was reflected by their unequal distribution in the sediments. Our results also suggest that the abundance of *tet*(W) in the sporobiota community is greater than *sul*1. This contrasts with previous studies quantifying ARG abundance within the total bacterial DNA, which have shown that the abundance of *sul* 1 was higher (or at least as abundant) than for other ARG, including *tet*(W) (*Munir, Wong & Xagoraraki, 2011*; *Czekalski, Gascón Díez & Bürgmann, 2014*; *Guo et al., 2014*; *Rodriguez-Mozaz et al., 2015*). In order to generalize this interpretation, the analysis of additional ARGs within the sporobiota needs to be considered in the future. This is even more relevant when taking into account the fact that the patterns of dissemination of ARG associated with the sporobiota appears to be unique, as suggested by the abundance of *tet*(W) across the area investigated. For instance, the low R^2 calculated for the linear regression between ARG abundance and distance (Fig. S2) demonstrated that ARG spatial distribution associated with the sporobiome is not only a function of distance to the outlet pipe (highest frequency of *tet*(W) found in the middle area). In contrast, a previous study on total microbial community DNA reported the highest relative abundance (copy number normalized by 16S rRNA gene copy numbers) of *tet*(W) in the vicinity of the wastewater discharge and a significant logarithmic decay in relative abundance with distance (*Czekalski, Gascón Díez & Bürgmann, 2014*). This suggests that *tet*(W) accumulation is specific to the sporobiota community. Interestingly, highest *tet*(W) frequencies were found among samples that coincided with a high level of particulate metal concentration in sediments (Figs. S1B; S3). Correlation between ARG and metal concentration has been observed in various environments (*Berg et al., 2010*; *Knapp et al., 2011*; *Ji et al., 2012*), including a previous study in Vidy Bay (*Devarajan et al., 2015*). However, it should be noted that not all the samples with high metal concentration have a high frequency of *tet*(W), suggesting that there might be other reasons for the correlation observed rather than co-selection. Moreover, co-selection is not expected for the sporobiota if the former is found in a metabolically inactive state (spore), but only in the case of the vegetative growing state.

Persistence and dissemination of ARG within the sporobiota appeared to be linked to a small number of species. The abundance of these ARG-bearing microorganisms in the human/animal microbiome and subsequent selection processes will strongly influence the potential of ARG to accumulate and spread in the environment. Our results show that endospore-forming Firmicutes play an important role in the environmental dissemination of certain ARG and thus, their potentially long-term persistence in the environment. Firmicutes are expected to be a major component of the sporobiota because of their ability to form endospores (*Schleifer, 2009*). In addition, Firmicutes might constitute a proxy for anthropogenic impact as they are common in the human microbiome (*Browne et al., 2016*). Moreover, this phylum includes a wide variety of pathogens, especially within *Clostridia* (*Popoff & Bouvet, 2009*).

Our results pointed to a *Clostridium* species as a potentially important vector for *tet*(W) dissemination and accumulation. This provides experimental support for the implication of the sporobiota and the sporobiome in the spread of antibiotic resistance (*Tetz & Tetz, 2017*; *Bengtsson-Palme, Kristiansson & Larsson, 2017*). The results are in

accordance with a previous study investigating the natural reservoirs of antibiotic resistance in the human gut, which revealed that *tet*(W) was preferentially present within Clostridiaceae, Ruminococcaceae, and Lachnospiraceae (*De Vries et al., 2011*; *Van Schaik, 2015*). Interestingly, the sample with the lowest relative abundance of Firmicutes was also the one with the lowest abundance/frequency of *tet*(W), suggesting that the abundance of Firmicutes in the sporobiome was a good predictor of *tet*(W) contamination.

Compared to *tet*(W), a larger fraction of the community was correlated to *sul*1. Notably among those were OTUs affiliated to Actinobacteria (Table 2). A recent study reported an increase in Actinobacteria abundance that correlated to an increase in *sul*1 frequency in activated sludge under tetracycline and sulfamethoxazole selection pressure, suggesting *sul*1 is well represented in Actinobacteria (*Zhang et al., 2016*). In addition to Actinobacteria, OTUs belonging to Firmicutes, Proteobacteria, and Saccharibacteria were also among the most correlated to *sul*1 frequency. This can be seen as a confirmation of the distribution of *sul*1 among a broad taxonomic range of bacterial clades, which is reflected in the sporobiome. The association of sulfonamide resistance with Class 1 Integrons and its transfer through horizontal gene transfer might explain this broad taxonomic distribution (*Aminov, 2010*).

The selection of ARG may already occur within human microbiota. It is now largely accepted that human intestinal bacteria not only exchange resistance genes among themselves, but can also interact with other bacteria in the colon (*Salyers, Gupta & Wang, 2004*). This makes the human gut a potential reservoir of antibiotic resistance genes for pathogenic bacteria (*Sommer, Dantas & Church, 2009*). Resistant spore-formers are then partially released with human feces and arrive in WWTPs, where (if in a vegetative state) they have the opportunity to mix and engage in horizontal gene transfer with other bacteria. If the final treatment is not sufficient to eliminate these organisms, they end up in the environment where they can interact with environmental organisms (*Baquero, Martínez & Cantón, 2008*).

CONCLUSION

The presence of ARG in the sporobiome highlights that it may be difficult to remove the legacy of resistance genes once released into the environment. Detecting ARG within this intrinsically long-lasting fraction of the microbial community also has important implications for the monitoring of ARG and the understanding of the processes explaining host-to-environment-to-host spread of antibiotic resistance. Notably, several studies have reported the transport and survival of thermophilic Firmicutes endospores in cold marine sediments, demonstrating their high dispersal and survival propensity (*Hubert, 2009*; *de Rezende et al., 2013*). Likewise, comparison with our previous study on a sediment core taken in the same basin but not directly influenced by the wastewater treatment discharge (Rhone catchment area; *Madueño et al., 2018*) also showed the enrichment of the sporobiota in the same resistance genes, providing further evidence that the sporobiota is a significant vector for ARG transport over considerable distance (Rhone river catchment). Moreover, the temporal analysis performed in our previous work suggests that the sporobiota

preserves the signature of ARG over long time scales (decades) (*Madueño et al., 2018*). Even if environmental conditions become harsh, this highly resilient fraction of the bacterial community can survive for long periods of time and may eventually re-enter humans via drinking water or other routes of contact, which may constitute an important long-term risk for human health.

ADDITIONAL INFORMATION AND DECLARATIONS

Funding

This work was supported by the Swiss National Science Foundation grant number CR23I2_162810. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Grant Disclosures

The following grant information was disclosed by the authors:
Swiss National Science Foundation: CR23I2_162810.

Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Christophe Paul performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
- Zhanna Bayrychenko and Sevasti Filippidou performed the experiments, analyzed the data, authored or reviewed drafts of the paper, approved the final draft.
- Thomas Junier performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, authored or reviewed drafts of the paper, approved the final draft.
- Karin Beck performed the experiments, contributed reagents/materials/analysis tools, authored or reviewed drafts of the paper, approved the final draft.
- Matthieu Bueche contributed reagents/materials/analysis tools, authored or reviewed drafts of the paper, approved the final draft.
- Gilbert Greub analyzed the data, authored or reviewed drafts of the paper, approved the final draft.
- Helmut Buergmann conceived and designed the experiments, analyzed the data, contributed reagents/materials/analysis tools, authored or reviewed drafts of the paper, approved the final draft.
- Pilar Junier conceived and designed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.

DNA Deposition

The following information was supplied regarding the deposition of DNA sequences:
Sequencing data was deposited to NCBI under the Bioproject accession number PRJNA396277.

Data Availability

The following information was supplied regarding data availability:

The raw data are included as Tables or as Supplemental Files.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.4989#supplemental-information.

REFERENCES

Adams V, Lyras D, Farrow KA, Rood JI. 2002. The clostridial mobilisable transposons. *Cellular and Molecular Life Sciences* **59**:2033–2043 DOI 10.1007/s000180200003.

Agergaard CN, Hoegh SV, Holt HM, Justesen US. 2016. Two Serious Cases of Infection with Clostridium celatum after 40 Years in Hiding? *Journal of Clinical Microbiology* **54**:236–238 DOI 10.1128/JCM.01938-15.

Albuquerque L, França L, Rainey FA, Schumann P, Nobre MF, Da Costa MS. 2011. Gaiella occulta gen. nov. sp. nov. a novel representative of a deep branching phylogenetic lineage within the class Actinobacteria and proposal of Gaiellaceae fam. nov. and Gaiellales ord. nov. *Systematic and Applied Microbiology* **34**:595–599 DOI 10.1016/j.syapm.2011.07.001.

Aminov RI. 2010. A brief history of the antibiotic era: lessons learned and challenges for the future. *Frontiers in Microbiology* **1**:1–7 DOI 10.3389/fmicb.2010.00134.

Angelakis E, Bibi F, Ramasamy D, Azhar EI, Jiman-Fatani AA, Aboushoushah SM, Lagier J-C, Robert C, Caputo A, Yasir M, Fournier P-E, Raoult D. 2014. Non-contiguous finished genome sequence and description of Clostridium saudii sp. nov. *Standards in Genomic Sciences* **9**:8 DOI 10.1186/1944-3277-9-8.

Baquero F, Martínez JL, Cantón R. 2008. Antibiotics and antibiotic resistance in water environments. *Current Opinion in Biotechnology* **19**:260–265 DOI 10.1016/j.copbio.2008.05.006.

Bartholomew JW, Paik G. 1966. Isolation and identification of obligate thermophilic sporeforming bacilli from ocean basin cores. *Journal of Bacteriology* **92**:635–638.

Bengtsson-Palme J, Kristiansson E, Larsson DGJ. 2017. Environmental factors influencing the development and spread of antibiotic resistance. *FEMS Microbiology Reviews* **42**:1–13 DOI 10.1093/femsre/fux053.

Berendonk TU, Manaia CM, Merlin C, Fatta-Kassinos D, Cytryn E, Walsh F, Bürgmann H, Sørum H, Norström M, Pons MN, Kreuzinger N, Huovinen P, Stefani S, Schwartz T, Kisand V, Baquero F, Martinez JL. 2015. Tackling antibiotic resistance: the environmental framework. *Nature Reviews Microbiology* **13**:310–317 DOI 10.1038/nrmicro3439.

Berg J, Thorsen MK, Holm PE, Jensen J, Nybroe O, Brandt KK. 2010. Cu exposure under field conditions coselects for antibiotic resistance as determined by a novel cultivation-independent bacterial community tolerance assay. *Environmental Science & Technology* **44**:8724–8728 DOI 10.1021/es101798r.

Berglund B. 2015. Environmental dissemination of antibiotic resistance genes and correlation to anthropogenic contamination with antibiotics. *Infection Ecology & Epidemiology* **5**:28564 DOI 10.3402/iee.v5.28564.

Bouki C, Venieri D, Diamadopoulos E. 2013. Detection and fate of antibiotic resistant bacteria in wastewater treatment plants: a review. *Ecotoxicology and Environmental Safety* **91**:1–9 DOI 10.1016/j.ecoenv.2013.01.016.

Browne HP, Forster SC, Anonye BO, Kumar N, Neville BA, Stares MD, Goulding D, Lawley TD. 2016. Culturing of "unculturable" human microbiota reveals novel taxa and extensive sporulation. *Nature* **533**:543–546 DOI 10.1038/nature17645.

Bueche M. 2014. *Spore-forming bacteria as indicators of pollution in sediments of Lake Geneva.* Switzerland: University of Neuchâtel.

Chen B, Liang X, Huang X, Zhang T, Li X. 2013. Differentiating anthropogenic impacts on ARGs in the Pearl River Estuary by using suitable gene indicators. *Water Research* **47**:2811–2820 DOI 10.1016/j.watres.2013.02.042.

Czekalski N, Berthold T, Caucci S, Egli A, Bürgmann H. 2012. Increased levels of multiresistant bacteria and resistance genes after wastewater treatment and their dissemination into Lake Geneva, Switzerland. *Frontiers in Microbiology* **3**:Article 106 DOI 10.3389/fmicb.2012.00106.

Czekalski N, Gascón Díez E, Bürgmann H. 2014. Wastewater as a point source of antibiotic-resistance genes in the sediment of a freshwater lake. *The ISME Journal* **8**:1381–1390 DOI 10.1038/ismej.2014.8.

De Rezende JR, Kjeldsen KU, Hubert CRJ, Finster K, Loy A, Jørgensen BB. 2013. Dispersal of thermophilic Desulfotomaculum endospores into Baltic Sea sediments over thousands of years. *The ISME Journal* **7**:72–84 DOI 10.1038/ismej.2012.83.

Devarajan N, Laffite A, Graham ND, Meijer M, Prabakar K, Mubedi JI, Elongo V, Piana PTM, Ibelings BW, Wildi W, Poté J. 2015. Accumulation of clinically relevant antibiotic resistant genes, bacterial load and metals from a freshwater lake sediments in central Europe. *Environmental Science & Technology* **49**:6528–6537 DOI 10.1021/acs.est.5b01031.

De Vries LE, Vallès Y, Agersø Y, Vaishampayan PA, García-Montaner A, Kuehl JV, Christensen H, Barlow M, Francino MP. 2011. The gut as reservoir of antibiotic resistance: microbial diversity of tetracycline resistance in mother and infant. *PLOS ONE* **6**:e21644 DOI 10.1371/journal.pone.0021644.

Dodd MC. 2012. Potential impacts of disinfection processes on elimination and deactivation of antibiotic resistance genes during water and wastewater treatment. *Journal of Environmental Monitoring* **14**:1754–1771 DOI 10.1039/c2em00006g.

Drudge CN. 2012. Prokaryotic horizontal gene transfer in freshwater lakes: implications of dynamic biogeochemical zonation. *Journal of Environmental Protection* **3**:1274–1294 DOI 10.4236/jep.2012.312181.

Federal Office of Public Health and Federal Food Safety and Veterinary Office. 2016. Swiss antibiotic resistance report 2016 usage of antibiotics and occurrence of antibiotic resistance in bacteria from humans and animals in Switzerland.

Guo X, Li J, Yang F, Yang J, Yin D. 2014. Prevalence of sulfonamide and tetracycline resistance genes in drinking water treatment plants in the Yangtze River Delta, China. *Science of the Total Environment* **493**:626–631 DOI 10.1016/j.scitotenv.2014.06.035.

Haller L, Tonolla M, Zopfi J, Peduzzi R, Wildi W, Poté J. 2011. Composition of bacterial and archaeal communities in freshwater sediments with different contamination levels (Lake Geneva, Switzerland). *Water Research* **45**:1213–1228 DOI 10.1016/j.watres.2010.11.018.

Herlemann DP, Labrenz M, Jürgens K, Bertilsson S, Waniek JJ, Andersson AF. 2011. Transitions in bacterial communities along the 2,000 km salinity gradient of the Baltic Sea. *The ISME Journal* **5**:1571–1579 DOI 10.1038/ismej.2011.41.

Heuer H, Smalla K. 2007. Manure and sulfadiazine synergistically increased bacterial antibiotic resistance in soil over at least two months. *Environmental Microbiology* **9**:657–666 DOI 10.1111/j.1462-2920.2006.01185.x.

Horn N. 1987. Clostridium disporicum sp. nov., a saccharolytic species able to form two spores per cell, isolated from a rat cecum. *IInternational Journal of Systematic Bacteriology* **37**:398–401 DOI 10.1099/00207713-37-4-398.

Hubert C. 2009. A constant flux of diverse thermophilic bacteria into the cold arctic seabed. *Science* **345**:1541–1544 DOI 10.1126/science.1174012.

Ji X, Shen Q, Liu F, Ma J, Xu G, Wang Y, Wu M. 2012. Antibiotic resistance gene abundances associated with antibiotics and heavy metals in animal manures and agricultural soils adjacent to feedlots in Shanghai; China. *Journal of Hazardous Materials* **235–236**:178–185 DOI 10.1016/j.jhazmat.2012.07.040.

Jin L, Kim KK, Baek SH, Lee ST. 2011. Kaistia geumhonensis sp. nov. and Kaistia dalseonensis sp. nov. two members of the class Alphaproteobacteria. *International Journal of Systematic and Evolutionary Microbiology* **61**:2577–2581 DOI 10.1099/ijs.0.028894-0.

Kenzaka T, Tani K, Nasu M. 2010. High-frequency phage-mediated gene transfer in freshwater environments determined at single-cell level. *ISME Journal* **4**:648–659 DOI 10.1038/ismej.2009.145.

Klieve AV, O'Leary MN, McMillen L, Ouwerkerk D. 2007. Ruminococcus bromii, identification and isolation as a dominant community member in the rumen of cattle fed a barley diet. *Journal of Applied Microbiology* **103**:2065–2073 DOI 10.1111/j.1365-2672.2007.03492.x.

Knapp CW, McCluskey SM, Singh BK, Campbell CD, Hudson G, Graham DW. 2011. Antibiotic resistance gene abundances correlate with metal and geochemical conditions in archived Scottish soils. *PLOS ONE* **6**:e27300 DOI 10.1371/journal.pone.0027300.

Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. 2013. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the miseq illumina sequencing platform. *Applied and Environmental Microbiology* **79**:5112–5120 DOI 10.1128/AEM.01043-13.

Lamendella R, Santo Domingo JW, Kelty C, Oerther DB. 2008. Bifidobacteria in feces and environmental waters. *Applied and Environmental Microbiology* **74**:575–584 DOI 10.1128/AEM.01221-07.

LaPara TM, Burch TR, McNamara PJ, Tan DT, Yan M, Eichmiller JJ. 2011. Tertiary-treated municipal wastewater is a significant point source of antibiotic resistance genes into Duluth-Superior Harbor. *Environmental Science & Technology* **45**:9543–9549 DOI 10.1021/es202775r.

Lennon JT, Jones SE. 2011. Microbial seed banks: the ecological and evolutionary implications of dormancy. *Nature Reviews Microbiology* **9**:119–130 DOI 10.1038/nrmicro2504.

Leser TD, Amenuvor JZ, Jensen TK, Lindecrona RH, Boye M, Moøller K. 2002. Culture-independent analysis of gut bacteria: the pig gastrointestinal tract microbiota revisited. *Applied and Environmental Microbiology* **68**:673–690 DOI 10.1128/AEM.68.2.673-690.2002.

Lozupone CA, Stombaugh JI, Gordon JI, Jansson JK, Knight R. 2012. Diversity, stability and resilience of the human gut microbiota. *Nature* **489**:220–230 DOI 10.1038/nature11550.

Madigan MT, Martinko J, Bender K, Buckley D, Stahl D. 2015. *Brock biology of microorganisms*. Boston: Pearson.

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 DOI 10.7717/peerj.4197.

Marti E, Variatza E, Balcazar JL. 2014. The role of aquatic ecosystems as reservoirs of antibiotic resistance. *Trends in Microbiology* **22**:36–41 DOI 10.1016/j.tim.2013.11.001.

McMurdie PJ, Holmes S. 2013. Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLOS ONE* **8**:e61217 DOI 10.1371/journal.pone.0061217.

Moore WEC, Cato EP, Holdeman LV. 1972. Ruminococcus bromii sp. n. and emendation of the description of Ruminococcus Sijpestein. *International Journal of Systematic Bacteriology* **22**:78–80 DOI 10.1099/00207713-22-2-78.

Mukhopadhya I, Moraïs S, Laverde-Gomez J, Sheridan PO, Walker AW, Kelly W, Klieve AV, Ouwerkerk D, Duncan SH, Louis P, Koropatkin N, Cockburn D, Kibler R, Cooper PJ, Sandoval C, Crost E, Juge N, Bayer EA, Flint HJ. 2018. Sporulation capability and amylosome conservation among diverse human colonic and rumen isolates of the keystone starch-degrader Ruminococcus bromii. *Environmental Microbiology* **20**:324–336 DOI 10.1111/1462-2920.14000.

Munir M, Wong K, Xagoraraki I. 2011. Release of antibiotic resistant bacteria and genes in the effluent and biosolids of five wastewater utilities in Michigan. *Water Research* **45**:681–693 DOI 10.1016/j.watres.2010.08.033.

Na G, Zhang W, Zhou S, Gao H, Lu Z, Wu X, Li R, Qiu L, Cai Y, Yao Z. 2014. Sulfonamide antibiotics in the Northern Yellow Sea are related to resistant bacteria: implications for antibiotic resistance genes. *Marine Pollution Bulletin* **84**:70–75 DOI 10.1016/j.marpolbul.2014.05.039.

Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, McGlinn D, Minchin PR, O'Hara RB, Simpson GL, Solymos P, Stevens MHH, Szoecs E, Wagner H. 2017. vegan: community ecology package. *Available at https://cran.r-project.org/web/packages/vegan/index.html*.

O'Neill J. 2015. Tackling a crisis for the health and wealth of nations. *The Review on Antimicrobial Resistance* DOI 10.1038/510015a.

Perry JA, Westman EL, Wright GD. 2014. The antibiotic resistome: what's new? *Current Opinion in Microbiology* **21**:45–20 DOI 10.1016/j.mib.2014.09.002.

Popoff MR, Bouvet P. 2009. Clostridial toxins. *Future Microbiology* **4**:1021–1064 DOI 10.2217/FMB.09.72.

Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO. 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Research* **41**:590–596 DOI 10.1093/nar/gks1219.

R Core Team. 2014. R: a language and environment for statistical computing. *Available at https://www.r-project.org*.

Rizzo L, Manaia C, Merlin C, Schwartz T, Dagot C, Ploy MC, Michael I, Fatta-Kassinos D. 2013. Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: a review. *Science of the Total Environment* **447**:345–360 DOI 10.1016/j.scitotenv.2013.01.032.

Roberts MC. 1996. Tetracycline resistance determinants: mechanisms of action, regulation of expression, genetic mobility, and distribution. *FEMS Microbiology Reviews* **19**:1–24 DOI 10.1016/0168-6445(96)00021-6.

Rodriguez-Mozaz S, Chamorro S, Marti E, Huerta B, Gros M, Sànchez-Melsió A, Borrego CM, Barceló D, Balcázar JL. 2015. Occurrence of antibiotics and antibiotic resistance genes in hospital and urban wastewaters and their impact on the receiving river. *Water Research* **69**:234–242 DOI 10.1016/j.watres.2014.11.021.

Saia FT, Souza TSO, Duarte RTD, Pozzi E, Fonseca D, Foresti E. 2016. Microbial community in a pilot-scale bioreactor promoting anaerobic digestion and sulfur-driven denitrification for domestic sewage treatment. *Bioprocess and Biosystems Engineering* **39**:341–352 DOI 10.1007/s00449-015-1520-6.

Salyers AA, Gupta A, Wang YP. 2004. Human intestinal bacteria as reservoirs for antibiotic resistance genes. *Trends in Microbiology* **12**:412–416 DOI 10.1016/j.tim.2004.07.004.

Sauvain L, Bueche M, Junier T, Masson M, Wunderlin T, Kohler-Milleret R, Gascon Diez E, Loizeau J-L, Tercier-Waeber ML, Junier P. 2014. Bacterial communities in trace metal contaminated lake sediments are dominated by endospore-forming bacteria. *Aquatic Sciences* **76**:33–46 DOI 10.1007/s00027-013-0313-8.

Schleifer KH. 2009. Phylum XIII. Firmicutes Gibbons and Murray 1978, 5 (Firmacutes [sic] Gibbons and Murray 1978, 5). In: De Vos P, Garrity GM, Jones D, Krieg N, Ludwig W, Rainey F, Schleifer K-H, Whitman W, eds. *Bergey's manual of systematic bacteriology*. Vol. 3. Dordrecht Heidelberg London New York: Springer, 19–1317.

Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG,

Van Horn DJ, Weber CF. 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and Environmental Microbiology* **75**:7537–7541 DOI 10.1128/AEM.01541-09.

Schmidt B, Mulder IE, Musk CC, Aminov RI, Lewis M, Stokes CR, Bailey M, Prosser JI, Gill BP, Pluske JR, Kelly D. 2011. Establishment of normal gut microbiota is compromised under excessive hygiene conditions. *PLOS ONE* **6**:e28284 DOI 10.1371/journal.pone.0028284.

Shoemaker WR, Lennon JT. 2018. Evolution with a seed bank: the population genetic consequences of microbial dormancy. *Evolutionary Applications* **11**:60–75 DOI 10.1111/eva.12557.

Sköld O. 2001. Resistance to trimethoprim and sulfonamides. *Veterinary Research* **32**:261–273 DOI 10.1051/vetres:2001123.

Sommer MOA, Dantas G, Church GM. 2009. Functional characterization of the antibiotic resistance reservoir in the human microflora. *Science* **325**:1128–1131 DOI 10.1126/science.1176950.

Spigaglia P, Barbanti F, Mastrantonio P. 2008. Tetracycline resistance gene *tet*(W) in the pathogenic bacterium Clostridium difficile. *Antimicrobial Agents and Chemotherapy* **52**:770–773 DOI 10.1128/AAC.00957-07.

Szczepanowski R, Linke B, Krahn I, Gartemann KH, Gützkow T, Eichler W, Pühler A, Schlüter A. 2009. Detection of 140 clinically relevant antibiotic-resistance genes in the plasmid metagenome of wastewater treatment plant bacteria showing reduced susceptibility to selected antibiotics. *Microbiology* **155**:2306–2319 DOI 10.1099/mic.0.028233-0.

Taylor NGH, Verner-Jeffreys DW, Baker-Austin C. 2011. Aquatic systems: maintaining, mixing and mobilising antimicrobial resistance? *Trends in Ecology and Evolution* **26**:278–284 DOI 10.1016/j.tree.2011.03.004.

Tetz G, Tetz V. 2017. Introducing the sporobiota and sporobiome. *Gut Pathogens* **9**:1–6 DOI 10.1186/s13099-017-0187-8.

Thevenon F, Adatte T, Wildi W, Poté J. 2012. Antibiotic resistant bacteria/genes dissemination in lacustrine sediments highly increased following cultural eutrophication of Lake Geneva (Switzerland). *Chemosphere* **86**:468–476 DOI 10.1016/j.chemosphere.2011.09.048.

Thorasin T, Hoyles L, McCartney AL. 2015. Dynamics and diversity of the "Atopobium cluster" in the human faecal microbiota, and phenotypic characterization of "Atopobium cluster" isolates. *Microbiology* **161**:565–579 DOI 10.1099/mic.0.000016.

Van Elsas JD, Bailey MJ. 2002. The ecolgy of transfer of mobile genetic elements. *FEMS Microbiology Ecology* **42**:187–197 DOI 10.1111/j.1574-6941.2002.tb01008.x.

Van Schaik W. 2015. The human gut resistome. *Philosophical Transactions of the Royal Society B: Biological Sciences* **370**:20140087–20140087 DOI 10.1098/rstb.2014.0087.

Vandewalle JL, Goetz GW, Huse SM, Morrison HG, Sogin ML, Hoffmann RG, Yan K, Mclellan SL. 2012. Acinetobacter, Aeromonas and Trichococcus populations dominate the microbial community within urban sewer infrastructure. *Environmental Microbiology* **14**:2538–2552 DOI 10.1111/j.1462-2920.2012.02757.x.

Walsh F, Ingenfeld A, Zampicolli M, Hilber-Bodmer M, Frey JE, Duffy B. 2011. Real-time PCR methods for quantitative monitoring of streptomycin and tetracycline resistance genes in agricultural ecosystems. *Journal of Microbiological Methods* **86**:150–155 DOI 10.1016/j.mimet.2011.04.011.

Wang RF, Cao WW, Cerniglia CE. 1997. PCR detection of Ruminococcus spp. in human and animal faecal samples. *Molecular and Cellular Probes* **11**:259–265 DOI 10.1006/mcpr.1997.0111.

Wang Z, Zhang X-X, Huang K, Miao Y, Shi P, Liu B, Long C, Li A. 2013. Metagenomic profiling of antibiotic resistance genes and mobile genetic elements in a tannery wastewater treatment plant. *PLOS ONE* **8**:e76079 DOI 10.1371/journal.pone.0076079.

Wunderlin T, Junier T, Roussel-Delif L, Jeanneret N, Junier P. 2013. Stage 0 sporulation gene A as a molecular marker to study diversity of endospore-forming Firmicutes. *Environmental Microbiology Reports* **5**:911–924 DOI 10.1111/1758-2229.12094.

Wunderlin T, Junier T, Roussel-Delif L, Jeanneret N, Junier P. 2014. Endospore-enriched sequencing approach reveals unprecedented diversity of Firmicutes in sediments. *Environmental Microbiology Reports* **6**:631–639 DOI 10.1111/1758-2229.12179.

Xu J, Xu Y, Wang H, Guo C, Qiu H, He Y, Zhang Y, Li X, Meng W. 2015. Occurrence of antibiotics and antibiotic resistance genes in a sewage treatment plant and its effluent-receiving river. *Chemosphere* **119**:1379–1385 DOI 10.1016/j.chemosphere.2014.02.040.

Yamada T, Sekiguchi Y, Hanada S, Imachi H, Ohashi A, Harada H, Kamagata Y. 2006. *Anaerolinea thermolimosa* sp. nov., *Levilinea saccharolytica* gen. nov,. sp. nov. and *Leptolinea tardivitalis* gen. nov., sp. nov., novel filamentous anaerobes, and description of the new classes *Anaerolineae* classis nov. and *Caldilineae* classis nov. in the bacterial phylum *Chloroflexi*. *International Journal of Systematic and Evolutionary Microbiology* **56**:1331–1340 DOI 10.1099/ijs.0.64169-0.

Zhang Y, Geng J, Ma H, Ren H, Xu K, Ding L. 2016. Characterization of microbial community and antibiotic resistance genes in activated sludge under tetracycline and sulfamethoxazole selection pressure. *Science of the Total Environment* **571**:479–486 DOI 10.1016/j.scitotenv.2016.07.014.

7 General Discussion

Foreword

In this chapter, I give a resume of the main findings of the preceding chapters and projects, and discussed the implications, importance and perspectives for future projects.

7.1 Main results

7.1.1 Foreword

In the preceding chapters, different terms have been used to describe the community obtained after the application of the spore-separation method: seed bank in “legacy”, sporobiota in “vidy”, spore-like or lysis-resistant organisms in “hamlet”. All these terms refers to the same resilient community, but have been adapted depending on reviewer’s comments. There is no evidence that the organisms found in this fraction of the community form structures that can be considered as “spores”. Therefore, the use of the term “spore” can be confusing. Indeed, this community is defined by its capacity to withstand the spore-separation method. Therefore, for the sake of clarity and homogeneity in the discussion, the terms lysis-resistant or spore-like structures will be used henceforth, except when the use of the term “spore” will be specifically chosen.

7.1.2 Cataloging the diversity of environmental lysis-resistant bacterial cells in environmental samples

In this chapter, we analyzed the lysis-resistant communities from different environments to offer an overview and increase our understanding of the diversity and distribution of potential spore-forming organisms in the environment. In addition, we assessed the efficiency of the spore-separation method by comparing the total and lysis-resistant communities in the same samples.

Main findings:

- The lysis-resistant community showed a unique signature compared to the total community, in samples from Lake Geneva (Switzerland).

- The genera not known to sporulate but found in the lysis-resistant community in high relative abundance, were among the least abundant in the total community, suggesting that contamination of the former by robust or abundant members of the total bacterial community is an unlikely explanation for the diversity observed in the lysis-resistant community.

- The lysis-resistant communities from the three types of environments were systematically dominated by Firmicutes, Actinobacteria and Proteobacteria (representing in average 86%), all known for containing representatives with the ability to produce spores, albeit through different mechanisms and with different properties.

- Among the wide diversity of taxa found in the lysis-resistant fraction of the community, many genera were hitherto not known to sporulate.

- The lysis-resistant community showed a geographic distribution pattern, challenging a hypothetical cosmopolitan distribution of the community forming lysis-resistant cells.

The results of this study demonstrated the efficiency of the spore-separation method for the enrichment of lysis-resistant structures. The diversity of the lysis-resistant community (at both phylum and genus level) and the high abundance of organisms not known to sporulate in this community, together with the observation of spore-like structures in genera also not known to sporulate suggests

that the ability to form spores or similar resting and durable cell structures is more widespread than previously considered.

7.1.3 Cross correlation of bacterial communities and geological proxies in paleoecology: a holistic approach for the study of past environmental history

In this chapter, we assessed the potential of using DNA from the total and lysis-resistant bacterial community as a complementary proxy for paleoecological studies. The study was conducted in the ephemeral Lake Liambezi, on the border between Namibia and Botswana.

Main findings:

- Climate reconstruction of the past 5500 years highlighted an alternation of dry and wet periods, reflected by variations in the lake regime (from fen to lake).

- Results showed variability in the lake regime between the three studied sites, explained by geomorphological characteristics of the lake.

- This spatial and temporal variability was reflected in both the geochemical parameters and the bacterial community.

- The grouping of samples (cluster analysis) based on the bacterial community composition was consistent with the grouping based on the visual characterization of the sediment and the geochemical analyses. This demonstrates that changes in the bacterial community (total and lysis-resistant) reflect changes in the environmental conditions.

- Analysis of the bacterial community and specific populations (sulfur-oxidizing and thermophilic bacteria) helped to correlate the three cores analyzed, and allowed to refine the age model.

- High abundance of numerous sulfur-oxidizing, sulfate-reducing and thermophilic bacteria pointed towards an active sulfur cycle and hydrothermal activity within the lake.

- Sulfate-reducing bacteria were detected mainly in the lysis-resistant fraction.

- Comparison of the total and the lysis-resistant communities, and concurrent analysis of the barium levels, suggested variability of the hydrothermal activity, and suggested that the hydrothermal source is in the north-basin of the lake, closer to the center coring site.

Overall, this study demonstrated the potential of using DNA from the total and the lysis-resistant community, as a complementary proxy for paleoecological studies. The use of an innovative multi-disciplinary approach, including bacterial DNA in complement to geochemical and sedimentological analyses, allowed the reconstruction of the hydrological regime and the climatic variations in Lake Liambezi (Namibia) during the past 5500 years.

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

In this chapter, we investigated the accumulation of antibiotic resistance genes (ARG) over time in DNA extracted from the total and lysis-resistant communities. The study was conducted in the Rhône Delta (Lake Geneva, Switzerland) using a sediment core covering the past 100 years.

Main findings:

- Two selected antibiotic resistance genes (*tet*(W) and *sul*1) were detected in DNA extracted from the total and lysis-resistant communities.

- Although the total abundance of ARG was higher in total community, their relative abundance was higher in the lysis-resistant fraction, indicating that the lysis-resistant DNA was enriched in ARG as compared to the total DNA.

- Different temporal accumulation patterns were observed for *tet*(W) and *sul*1, reflecting the timeline of the medical use of their related antibiotic.

- Correlation tests between ARG and OTUs resulted in a low number of high correlations, suggested that only few OTUs possess ARG.

- Higher number of high correlations for *sul*1 compared to *tet*(W) suggested that more OTUs possess *sul*1 than *tet*(W).

- The taxonomic distribution of ARG appeared to be heterogenous: *tet*(W) accumulation highly correlated with changes in the abundance of Firmicutes, while *sul*1 accumulation correlated with a wide range of taxonomic groups.

Overall, the results suggest that the relative abundance of ARG in the lysis-resistant fraction constitute a good proxy to evaluate the accumulation of ARG in the environment in relationship with the historical use of two specific antibiotics.

7.1.5 Dissemination of antibiotic resistance genes associated with the sporobiota in sediments impacted by wastewater

In this chapter, we investigated the impact of wastewater treatment plant (WWTP) releases on the abundance of ARG in sediments of the Vidy Bay (Lake Geneva, Switzerland).

Main findings:

- WWTP was identified as a main source of ARG, illustrated by the detection of ARG in all samples, the decreasing abundance/frequency of ARG with increasing distance to the WWTP outlet, and the correlation between ARG levels and concentrations of C_{org}, N_{tot} and DNA, all indicators of wastewater discharge.

- The different distribution patterns for *tet*(W) and *sul*1, and their correlation with different taxonomic groups, suggested that both ARG were differently enriched in lysis-resistant community.

- *tet*(W) frequency (copies/ng DNA) correlated with OTUs belonging to Firmicutes (*Clostridium* and *Ruminococcus)*, while *sul1* correlated with a large taxonomic spectrum of OTUs.

- *Clostridium* sp. was found in high relative abundance and was highly correlated with *tet*(W) frequency, suggesting it might be a potential vector for *tet(W)* dissemination.

- The low relative abundance of OTUs correlating with *sul1* suggested that the *sul1*-associated lysis-resistant fraction might have low impact on ARG dissemination.

Overall, the results of this study highlighted the impact of wastewater releases on the dispersal of ARG in the environment. The high survival and dispersal rate of spores and other lysis-resistant structures raises serious concerns in terms of dissemination and persistence of ARG in the environment.

7.2 Discussion

7.2.1 General considerations

The environmental significance of spores and spore-forming bacteria remains widely unexplored, with most of the knowledge on spore-formers and sporulation coming from model organisms studied in the laboratory (Nicholson et al., 2000; Nicholson, 2002). Little is known regarding the ecological role of spores and spore-formers, their significance in population dynamics and/or their interactions with other living populations. This lack of knowledge can be attributed, at least partly, to methodological considerations. The study of spores in the environment using genomics and other PCR-dependent methods is challenging, due notably to methodological issues and the difficulty to access spore DNA and the impossibility to distinguish it from vegetative cell DNA. The recent development of a series of molecular tools dedicated to the study of spores and spore-forming bacteria (Bueche et al., 2013; Wunderlin et al., 2013, 2016; Junier et al., 2015) filled, at least partly, this methodological gap. The application of these innovative methods provided new insights, not only about their possible use as a paleoecological proxy (Wunderlin et al., 2014a), but also about the unsuspected diversity of spore-formers in sediments (Wunderlin et al., 2014b).

7.2.2 Diversity of spore-formers

Analysis of the bacterial community from the different projects conducted revealed an unsuspected diversity of taxa in the lysis-resistant fraction of the community. Firmicutes, Proteobacteria and Actinobacteria were systematically the most abundant phyla, which is not surprising since the ability of some members of these phyla to form spores is well documented (see introduction). However, the analysis of the community at the genus level revealed that many of the detected organisms, including some with very high abundance, are not known to form spores. One can argue that this is the result of a methodological issue, namely the method for the separation of spores failing to remove the vegetative cells. Therefore, the non-spore-forming organisms detected in the lysis-resistant fraction of

the community might be the result of a contamination. However, a close analysis of the data strongly suggests that this not the case.

When comparing the total and the lysis-resistant communities (from Lake Geneva and Lake Liambezi studies), it appears that the separation treatment induced a clear change in the bacterial community. Firmicutes and Actinobacteria significantly increases, while other taxa such as Bacteroides, Chlorobi and Parcubacteria tend to disappear from the community, corroborates the current knowledge about bacterial spore-formers. However, at OTU level, it appears that in most cases, the most abundant OTUs are different in the total and lysis-resistant communities. More importantly, the most abundant OTUs not known as spore-formers found in the lysis-resistant fraction (i.e. *Mesorhizobium, Gaiella, Rhizobium*, *Arthrobacter*, *Acidothermus*, *Fonticella*) are, in most cases, not among the most abundant OTUs of the total community. Considering the detection of non-spore-formers in the lysis-resistant fraction as the result of contamination, one can expect to find the most abundant OTUs of the total community as the main contaminant. The simple fact that an OTU have a higher relative abundance in the lysis-resistant fraction than in the total community indicates that this OTU has been enriched compared to other OTUs following the separation of spore treatment. This demonstrates an ability of withstand degradation, at least above the average, demonstrating that separation of spore method has a different impact depending on taxa, and acts as a selective process. We do not pretend to remove completely the vegetative cells. In fact, an enrichment between 84 and 90% was reported for the method (Wunderlin et al., 2014b). However, we can assert that OTUs found in high relative abundance in the lysis-resistant fraction present some ability to withstand degradation.

From an evolutionary point of view, the ability to form spores is of great advantage in terms of survival, because of the high dispersal rate potential and the ability to withstand unfavorable harsh conditions (Lennon & Jones, 2011; Shoemaker & Lennon, 2018). Therefore, the hypothesis that this feature evolved widely among Bacteria is far from unlikely. Moreover, the fact that spores have not been reported for a species, genus or group of organisms does not constitute a proof of the inability of the group to produce spores. For instance, a recent study has reported spore formation for members of the genus *Ruminococcus*, which was considered as non-sporulating until then (Mukhopadhya et al., 2018). It is likely that this is also the case for other taxa. The question is to which extend is this the case?

Our studies suggest that the ability to form spores is widespread among bacteria, in species/genera, but also in phyla that are not recognized as spore-formers. However, the method used does not allow to demonstrate the production of spores by the identified organisms. Only the direct observation of the formation of spores in each of these taxa would constitute an irrefutable proof. Conversely, the absence of observation is not a proof of the inability to form spores. In fact, the “real” concern is about the use of the term “spore”, which refers to a wide variety of structures produced by various organisms among both prokaryotes and eukaryotes. In Bacteria, spores refer to specific structures associated to different groups of organisms: endospores for Firmicutes, exospores for Actinobacteria, myxospores for Myxobacteria or akinetes for Cyanobacteria (Barton, 2005).

Demonstrating the formation of spores for each species/taxon individually appears not only unrealistic, but is beyond the scope of this project. To be a suitable as a proxy, the selected structure has to be preserved in sedimentary records. This is more a functional than a structural trait. Given the nature of the spore treatment (lysozyme, NaOH, heat, DNase), we assume that cells that are able to withstand this treatment are also likely to be preserved from degradation in natural environments.

7.2.3 Lysis-resistant versus total community

The idea of using DNA from spores (or spore-like structures) as biological proxy as an alternative to total DNA, came from the hypothesis that DNA in vegetative cells is subjected to degradation while DNA within spores might be preserved for extended times. Moreover, DNA degradation is considered to be taxon-dependent and related to environmental conditions (Boere et al., 2011), suggesting that a change in the community might reflect this susceptibility to degradation more than a change in the environmental conditions.

Our results indicate that total DNA indeed decreases with depth (and time), while spore DNA is more stable in time, suggesting spore DNA is more resistant to degradation. In Lake Geneva, the total DNA yields were 2-3 order of magnitude higher than lysis-resistant DNA, while in Lake Liambezi, the difference was smaller (~1 order of magnitude). This can probably be explained by the different time scales covered by the cores (~100 years for Lake Geneva, up to 5'500 years for Lake Liambezi), and/or a higher degradation rate in Lake Liambezi. In both cases, total DNA decreased with depth, but the decrease was higher in Lake Geneva, suggesting that the main part of vegetative cells might be degraded quickly (decade scale) after deposition, the decrease slowing down afterwards. Although the decay less pronounced in Lake Liambezi, this can be due to the resolution of the sampling. Possibly sediment from the last century was not retrieved, and therefore, the exponential decay was not seen. For the lysis-resistant fraction, data obtained from Lake Geneva and Lake Liambezi showed similar DNA yields (same order of magnitude). The decrease observed in Lake Geneva suggests that lysis-resistant structures are also subject to degradation, but to a smaller extend than vegetative cells. For older samples (Lake Liambezi), lysis-resistant DNA yields are relatively constant. These results also have to be mitigate because these interpretations does not take into account the variations between and within cores: sedimentation rate, variability in organic matter production and exogenous inputs, microbial activity in the sediment, etc… To noteworthy indicating that the total DNA comprised not only bacterial DNA, leading to an overestimation when comparing with lysis-resistant DNA. Moreover, all the calculations were made based on the quantification after DNA extraction, and it is likely that the extraction efficiency is variable, depending on the sample characteristics and some random effect probably due to the heterogeneity of the samples and the reproducibility due to the technician.

Our results are consistent with previous studies, which reported a better conservation of spores in sediment. Analyzing the same sediment core and samples from Lake Geneva using a quantitative PCR approach, the abundance of endospore-forming bacteria (copies number of *spo0A* gene) showed to be relatively constant with depth, while total DNA and total bacteria (copies number of 16S rRNA gene) showed an exponential decay. Although this study did not isolate the lysis-resistant fraction, but targeted the fraction able to form endospores, whether it is vegetative cells or endospores, both studies highlighted the preservation ability of endospores or other lysis-resistant structures. Another study conducted in the sediments of Lake Constance and using a cultural approach showed that viable heterotrophic bacterial cells were mainly present under the form of spores below 25 cm, which correspond to ~170 years (Rothfuss, Bender & Conrad, 1997). In addition, spore abundance decreased exponentially in the first 6 meters of the sediment core. Such an exponential decrease was not observed in our samples. This can be due to the different approach used (cultural vs molecular). While the cultural approach took into account the viable cells, the molecular approach we used does not make this distinction. Therefore, spores can be preserved in sediments, but their ability to resuscitate decrease with time. If the bacterial cells were mainly present under the form of spores or spore-like structures in old sediments, as suggested by Rothfuss et al. (1997), one can expect to detect the signature of lysis-resistant community in the total community. Indeed, total and lysis-resistant

communities might be more similar in lower sediment compared to upper sediment. This does not seems to be the case in Lake Liambezi. Older samples are not more similar than latest ones, except in the north core (as shown by the distance matrix and the PCoA), suggesting that the signal of vegetative cells overcomes those of the lysis-resistant cells in the total community, even in old samples with lower DNA yields. This also suggests that the DNA yields is not a good estimator of the ratio vegetative/lysis-resistant cells. This can also be the result of the microbial activity in the sediment, affecting more strongly the active than the inactive community.

Despite these uncertainties regarding the preservation of DNA/vegetative cells, our results suggest that bacterial DNA extracted from the total community can be used as a proxy. Even in old samples (up to 5'500 years in Lake Liambezi), DNA yields were sufficient for DNA-based analyses such as HTS sequencing or qPCR quantifications. In most cases DNA yields for the total DNA was also higher than for lysis-resistant DNA. Moreover, the grouping of samples based on both total and lysis-resistant communities (cluster analysis) were consistent with the grouping of samples based on the visual characterization of the sediment cores and the geochemical proxies. This suggests that the change in the community reflects changes in the environment, and probably not an artefact produced by differential degradation. Why then to use lysis-resistant DNA if the total DNA can be used instead? The method to extract the lysis-resistant fraction is more complicate, expensive, and time-consuming. Moreover, DNA yields are lower, and lysis-resistant community might not reflect the activity of bacteria and thus, not reflect environmental conditions.

In fact, these two proxies may be complementary. Considering our results from Lake Liambezi, although the clustering of samples based on both communities were consistent, the lysis-resistant community reflected better the changes in the environmental conditions than the total community did. This was interpreted as a possible consequence of the microbial activity in the sediment. Likewise, the study conducted in Lake Liambezi also showed that environmental conditions, biological processes, trends or extreme events were detected better or only in one of the fractions. Some groups of organisms appeared to be mainly detectable in the lysis-resistant fraction, such as sulfate-reducing and mammals-associated bacteria. On the contrary, sulfur-oxidizing bacteria were more diverse and detected in higher abundance in the total fraction, since the most abundant sulfur-oxidizing bacteria are not known to form spores.

Importantly, the omission of one fraction could lead to misinterpretation. In the lysis-resistant fraction, the sulfur-oxidizing bacteria were not only detected at lower abundance, their abundance pattern was also different. In the upper half of the center core, sulfur-oxidizing bacteria were almost absent, while they were highly abundant in the total community, reaching up to 30% of the total community. This could lead to completely different interpretation about the environmental conditions and/or the availability of sulfur for example. Furthermore, the lysis-resistant community might provide additional information about phenomena or events occurring in the watershed, while the total community is more likely to reflect the environmental conditions reigning in the lake. Each community appears to provide relevant and complementary information and their concurrent use allow to enhance our understanding of the studied system.

7.2.4 First detection of ARG in DNA extracted from lysis-resistant cells and validation of the method

Although the presence of genes conferring resistance to antibiotics has been previously shown in spore-forming bacteria (Tetz & Tetz, 2017 and references therein; see introduction for more references), the two studies conducted (chapter 5-6) provide the first evidence for the detection of ARG directly from spores or other lysis-resistant structures. This is of great interest since spore-formers have been recently shown to be prevalent in the human microbiome (Browne et al., 2016; Forster et al., 2019), and it is supposed to have a significant role in the dissemination of ARG. Spores are not only a potential vector for ARG dissemination due to high dispersal rate. They are also more likely to acquire a beneficial mutation (Levin-Reisman et al., 2017) or resistance genes because they can survive antibiotic treatment and co-evolve with ARG-bearing organisms, highlighting the importance of our findings. Although resistance to antibiotics is a growing public health concern in medical environment, little is known about the environmental significance of ARG and the consequences of releasing ARG and ARG-bearing organisms in the environment. Likewise, much less is known about the role of spore-forming bacteria in ARG dispersal and accumulation. Although several authors have recently pointed their potential important role (Bengtsson-Palme, Kristiansson & Larsson, 2017; Tetz & Tetz, 2017; Shoemaker & Lennon, 2018), no study (to the best of our knowledge) has investigated its environmental significance, probably due to the poor understanding of this fraction and the lack of adapted tools. Therefore, the development and use of tools allowing the study of this specialized fraction of the community is essential to assess its role in the dissemination of antibiotic resistance.

Using the newly developed method for the study of endospores, we demonstrated the potential to use spore or lysis-resistant DNA for tracking ARG in the environment. The results of our studies demonstrated the relevancy of this novel approach, and showed its applicability as a proxy for:

- Monitoring the environmental pool of ARG associated to this highly persistent, resilient and mobile fraction of the community.

- Assessing the impact of WWTP and other sources of ARG (for example animal husbandry) on the dispersal of ARG.

- Reconstructing the accumulation of ARG over time, and evaluating the effect of change in the use of antibiotics on the pool of environmental ARG.

- Identifying potential ARG-carriers within the spore community.

Increased knowledge and a global vision of the environmental pool of ARG, dispersal rate and dissemination vectors, and the “long-term” effect of antibiotic usage on the fate of ARG, may help to develop adequate policies for a reasonable usage of antibiotics.

7.2.5 ARG in the total vs lysis-resistant community and abundance vs frequency

Our postulate was that DNA extracted from spores constitutes a better proxy than total DNA, due do the degradation of DNA, selective or not, inducing a potential bias in the detected ARG levels, which is not the case with the DNA extracted from spores. The result of our study in the Rhône Delta in Lake

Geneva (chapter 5) brought other arguments in favor of the use of DNA extracted from the spore or lysis-resistant cell fraction. First, for both studied genes, the quantification based on total DNA revealed low numbers of ARG copies, which were close to the detection limit of the qPCR. Normalized to the amount of DNA (frequency=ARG copies/ng DNA), both genes are almost undetectable on the whole profile of the core, except for the last 10 years for *sul*1. Second, the amount of required DNA template used for qPCR (determined by successive dilutions on a selection of DNA extracts) is larger for the total DNA than for the lysis-resistant DNA, suggesting a lower ratio of targeted DNA in the total DNA.

Once the total amount of DNA extracted per gram of sediment is considered, ARG abundance is higher in the total DNA. This was expected given the low amount of DNA retrieved from the spore-separation method. However, ARG frequency is higher in the lysis-resistant fraction, suggesting an enrichment of ARG in the latter, which is highly unexpected result. However, we must be careful while interpreting these results. First, the efficiency of the qPCR depends on many factors, such as dilution and purity of the DNA. Likewise, the proportion of the targeted DNA within the total DNA also influence amplification efficiency. Based on the results of the qPCR optimization, different concentrations of DNA were used for the total and lysis-resistant DNA. Therefore, the direct comparison of the abundance (both total and relative) obtained from the total and the lysis-resistant DNA might be biased. Moreover, ARG frequency was calculated by normalizing ARG copies by the amount of DNA. In the case of lysis-resistant structures, the entire DNA extracted should belong to bacteria, while for the total DNA other taxa might be represented, leading to an underestimation of the ARG frequency in the total bacterial DNA. Hence, a normalization based on the number of 16S rRNA gene copies or *rpoB* could help to overcome or at least limit this bias. Unfortunately, all the remaining DNA was used for the sequencing and we could not proceed to the quantitation of these additional markers.

ARG enrichment of the lysis-resistant fraction suggests a propensity of spore-formers to acquire ARG compared to non-spore-formers. This could be the results of a selective process. Since spore-formers can survive antibiotic stress, they are more likely to acquire a resistance gene simply because they survive long enough to undergo a beneficial mutation (Levin-Reisman et al., 2017). Moreover, supposing that the human microbiome might be enriched in both antibiotic resistant organisms and spore-formers, the isolation of spores would inevitably lead to an enrichment in ARG. An alternative explanation would be the heterogeneous distribution of ARG among taxa. If an ARG is preferentially present in a taxon with sporulation abilities, then the rise or the decline of this taxon would lead to the subsequent change in the associated-ARG frequency, even if the factors selecting are independent of antibiotic resistance. It remains difficult to discriminate these different explanations, because it is difficult to determine if ARG frequency increase because a population becomes more resistant, or because a resistant population becomes more abundant. Maybe the frequency within a population does not change, but the relative abundance of this population increases. However, determining if the enrichment in ARG is due to a selective process within or across taxa (selection of ARG-bearing organism within taxa, or selection of ARG-bearing taxa) is impossible for our data and the methods used. *In vivo* experiments (see following section) are required to help addressing this question.

Comparison of abundances and frequencies was also very informative. Frequency has the advantage to eliminate the effect of the DNA amount retrieved from the different samples, therefore removing potential bias due to variable efficiency of the DNA extraction method (possibly due to sediment nature), or variable organic matter content. It is then suitable when investigating the enrichment in ARG rather than their “true” abundance. Interestingly, both the frequency of *tet*(W) and *sul*1 showed particular accumulation patterns, correlating with the historical use of their related antibiotic, which

was not reflected in the accumulation pattern based on ARG abundance (see chapter 5). In the study conducted in the Vidy Bay, *sul*1 abundance and frequency as well as *tet*(W) abundance correlate with the distance to the WWTP. However, the frequency of *tet*(W) showed a particular pattern, being associated to samples with high trace metal concentrations. These results suggest that frequency might be a better measure to highlight events or relationships that are not visible with abundance. In fact, abundance mostly represent the amount of DNA retrieved from the sediment and thus is rather a measure of the productivity and/or deposition rate. As a precision, it is obvious that for a study aiming to evaluate the global pool of ARG, then ARG abundance in the total community would be more appropriate.

7.2.6 Heterogeneity of ARG distribution among taxa

The two studied ARG were detected in the lysis-resistant fraction but appeared to be differently associated with different groups of organisms, suggesting ARG are not homogeneously distributed among bacterial taxa. Results indicated an association between *tet*(W) resistance and Firmicutes, confirming previous studies (de Vries et al., 2011; van Schaik, 2015). On the contrary, *sul*1 was associated with a wide range of taxa, suggesting a wide distribution among various taxa. These results were expected in a certain way. Given that *sul*1 is commonly associated with class-1 integrons, it may favor its transfer among taxa (Aminov, 2010). The heterogeneous distribution of ARG within taxa was also reflected in the ARG levels (abundance/frequency) measured. The same order of magnitude for *tet*(W) and *sul*1 abundance were observed in samples from the Rhône Delta (chapter 5). But in the Vidy Bay (chapter 6), the abundance and frequency of *tet*(W) is higher than for *sul*1. This can be explained by the community structure from the Vidy Bay, which are largely dominated by Firmicutes. This is probably due to their prevalence in the human microbiome (Browne et al., 2016; Forster et al., 2019) and to the footprint of humans in the communities surrounding a WWTP.

This heterogeneous distribution of ARG among taxa is important since different bacterial taxa might behave differently in the environment, thus, having an impact on the dissemination potential of ARG. The ability of these human-associated microorganisms to survive in natural environments is a determining factor to establish the potential threat they represent in terms of dispersal, dissemination, gene exchange rate, re-introduction, etc… Horizontal gene transfer (HGT) has been suggested as the main pathway for spreading of the ARG in the environment (Pruden et al., 2006). However, for gene exchange to occur, strains have to share the same habitat, at least temporarily. Moreover, in the case of spore-forming bacteria, they have to be active. One can argue that these human-associated populations are not adapted to natural environments and might remain in a spore state (if they can), or decline and die. In addition, HGT is more likely to occur between closely phylogenetically related organisms. Therefore, HGT is supposedly more frequent between pathogens and human-associated organisms from the microbiota, than between human-associated organisms and environmental strains (Bengtsson-Palme, Kristiansson & Larsson, 2017).

However, little is known about the fate of human-originated microorganisms when released in the environment. Although spores are inactive, implying that no transfer is possible from these organisms, it is difficult to determine where sporulation occurred and what are the factors that trigger sporulation/resuscitation in human and natural environments. Likewise, the extent to which the human microbiota can survive and grow in natural environments, notably in hotspots (sediments and biofilms) where activity and gene exchange rate are enhanced (Taylor, Verner-Jeffreys & Baker-Austin, 2011; Marti, Variatza & Balcazar, 2014), has not been established. Giving that the diversity of spore-

formers is probably largely underestimated, their ecological significance and population dynamics in natural environments remains widely unknown.

7.3 Perspectives

7.3.1 Diversity of spore-forming organisms

Although the purpose of this project was not to evaluate the diversity and prevalence of spore-formers among the domain of Bacteria, our results revealed an unsuspected diversity in the lysis-resistant community. This has great implications in terms of evolution and supports the idea of a widespread functional trait that evolve early in the life history, emerging in a common ancestor prior to its radiation among taxa (Tocheva, Ortega & Jensen, 2016). However, as mentioned above, our results do not provide proofs on the formation of spores in the taxa detected. We can only assert that these enriched taxa resist the separation of spore treatment, at least more than average.

For proving the formation of spores in these taxa, each organism should be isolated and cultivated separately. Only the direct observation of the formation of spores can be considered as a proof. However, such an approach is unrealistic if one want to assess the ability of forming spores in each taxon, moreover many of these organisms being uncultivable. An intermediate option would be to concentrate the effort on some taxa, for example those found in high abundance in the lysis-resistant community. If one can demonstrate the formation of spores in most of the tested taxa, that would constitute a good indication that spore-separation really enrich spores, and not only some lysis-resistant structures. An alternative to the culture-based approach would be to look for genes associated to sporulation in genomes available in databases. Model organisms are often used to predict the ability of forming spores of other organisms, although this limits the investigations to the known regulatory pathways of sporulation. Once again, the presence of genes associated to sporulation does not constitute a proof in itself, but this can drive the investigations towards organisms that are more likely to produce spores. The whole genome sequencing from environmental samples also constitute another alternative option. The actual limitations of the method (high quantity DNA needed, few genomes reconstructed) would restrict the analysis to a small number of samples and genomes, and would not be representative of the diversity found in our samples. However, the rapid development associated to metagenomics can sustain the hope that such studies can be performed in the next future.

7.3.2 Environment and time scale

Although the use of total DNA and DNA from the lysis-resistant organisms showed its relevance in Lake Liambezi, this constitutes only one step forward in the evaluation of its potential use as a proxy for paleoecology. Supplementary studies are needed to give an overview of its potential applicability.

The response of an ecosystem to environmental changes and fluctuations is complex and depends on multiple parameters (climate, geomorphology, biodiversity, among others). The variability of the bacterial community is related to the variability of the environmental conditions; "*Microbial community in a certain habitat is shaped by the interaction of microbes and the environment, while the microbes also modify the environments by their metabolism, especially the recycling of the nutrients*"

(Chen et al., 2015). We can assume that a fluctuating environment will induce higher variability in the community. The variability of the bacterial community, and at which extend it reflects the environmental changes might be variable, depending on the type of ecosystem under investigations and its adaptability/resilience capacity. Therefore, we aim to broaden the application of this novel approach and test its potential for paleoecological studies in other aquatic systems, from various environments.

Another parameter of main importance is the time scale of the study, which depends on the length of the core (and the time covered), the resolution of the sampling, and the sedimentation rate. While our study in Lake Geneva was based on a sediment core of ~1 meter covering the last 100 years, the center core from Lake Liambezi covered 5500 years but the length of the core was only 50 cm. It appears obvious that the environmental changes cannot be investigated at the same time scale, and that seasonal or annual variations of the environmental conditions may not be detected in the latter. For example, several studies showed high seasonal variability of the bacterial community in sediment from lakes of rivers (Bucci et al., 2014; Zhang et al., 2019). However, if the sedimentation rate is low, the different communities that have developed at different times (seasons) might be mixed in the same sediment layer. In addition, another study showed a reoccurrence of the bacterial community over years in river sediments (Hullar, Kaplan & Stahl, 2006). Considering a low sediment rate, we could easily conclude from this reoccurrence that the community is stable over time, missing the seasonal variations due to the condensate sediment layers.

In Lake Liambezi, deductions on environmental conditions based on the community composition were difficult to establish for several reasons: (i) low variability of the community, (ii) condensate layers (low sedimentation rate), and (iii) the resolution of the sampling. From the climatic perspective, the low variability of the community was interesting and showed that the system was relatively stable over time, despite changes in the hydrological regime. On the contrary, the seasonality of the system (alternation of wet/dry seasons) was not recorded in the microbial community, due to the condensate layers and the resolution of the sampling. This illustrates the importance of the time scale, and determining carefully the sampling strategy in function of the problematic under investigation. Therefore, it would be of great interest to apply our approach to different problematics, implying different time scales.

Lake Fagnano, located in “Tierra del Fuego” (Patagonia), is one of the ecosystems we aim to investigate. A series of studies based on sediments and seismic analyses allowed for the reconstruction of the past climate and the tectonic activity on the island of Tierra del Fuego during the Holocene (Waldmann et al. 2010; Moy et al. 2011; Waldmann et al. 2011). The most recent of these studies presented a multidisciplinary approach for the reconstruction of the paleoenvironmental conditions in Tierra del Fuego, allowing for a better understanding of the environmental changes under the influence of the Southern Hemisphere Westerlies wind stream (SHW) in the region, and the impact on the surrounding vegetation (Waldmann et al. 2014).). In particular, the use of different biological proxies in this study, palynomorphs analyses (pollens, spores, and algae) associated to palynofacies offers very interesting data for comparison with bacterial communities and assessing the validity of our method.

On the contrary, working at a much smaller time scale, our study in the Vidy Bay (Lake Geneva) showed a high proportion of human-associated bacteria in sediments impacted by wastewater releases. In addition, results of Lake Liambezi also exhibits high abundance of mammal-associated organisms in several layers. This suggests that spores could be used for evaluating the anthropogenic impact in

modern ecosystems. Such a proxy could help to establish statements of ecosystem health, and the implementation of protective measures.

Considerations about DNA

The time scale also have main implications for the conservation of the DNA. The more the DNA is ancient, the more a bias due to selective degradation is likely to appear. In Lake Liambezi, the total DNA showed to be appropriate for the climatic reconstruction over the last 5500 years, in terms of both quantity and quality. However, what about studies at smaller or bigger time scale? In a previous study in Lake Geneva (using the same core used in our study), DNA showed an exponential decay in the first decades following deposition (Wunderlin et al., 2014a). Therefore, a selective degradation of the DNA might affect the total community, possibly blurring the seasonal signal. Likewise, the degradation of DNA may be too important in older sedimentary records, or in different environments, limiting its availability and increasing the bias.

Enlarge the application of our innovative approach to various types of ecosystems, and evaluate its relevancy in different environments, at different time scales, is necessary to demonstrate the utility of the method and define the scope of its applicability.

7.3.3 The origin of spores

One main concern about the possible use of spores and spore-like structures as a paleoecological proxy is their origin. Previous studies have shown their exogenous origin due to their high dispersal capacity (Bartholomew & Paik, 1966; de Rezende et al., 2013). In Lake Liambezi, the populations found in the lysis-resistant community suggested both an endo- and exogenous origin. This consideration is of main importance if one want to interpret correctly the community composition, and its relation to the ecosystem; this has considerable implications for the spatial scale of the study. Does the lysis-resistant community found in the sediment reflect the environmental conditions reigning in the lake, or is it representative of the whole watershed?

Spores, and subsequently by extension lysis-resistant structures, are assumed to be inactive. Therefore, we may wonder whether their analysis inform about the environment they are found in. To better interpret the composition and the changes in the lysis-resistant community in the sediments, a better knowledge of its community dynamic is needed. Although sporulation/reactivation of model organisms have been observed and studied in laboratory, little is known about the response of sporulating organisms to environmental changes and their role in the shaping and adaptability of the community. Bacterial spores are considered to have a prominent role in the resilience of ecosystems after perturbation, due to their ability to colonize new habitats. However, no study demonstrated the link between dormancy and the community dynamic (Lennon & Jones, 2011).

One of our next objectives is to investigate the origin of lysis-resistant structures, and follow the evolution of the lysis-resistant community in their response to environmental change. The Jöri lakes, located in the Jöri catchment in the Swiss Alps (Canton Graubünden) was selected as model environment. Initially formed by the Jöri glacier, the catchment currently encompasses 24 small and medium cascading lakes (at sampling time), distributes on different sub-catchments (their exact number changes depending on the source; Hinder et al. 1999; Yuhana et al. 2006). Some of these lakes are relatively ancient (several thousand years old) while others have been formed more recently during the last 150 years with the progressive retreat of the glacier (Yuhana, Horath & Hanselmann, 2006).

Cascading lakes from the same sub-catchments might be viewed as the same lake at different stage of evolution, and thus can be considered as natural chronological records. This constitutes a perfect model for studying the establishment of communities of spore-forming bacteria in sediments, and their evolution over time. We can suppose that, if the lysis-resistant community reflect the environmental conditions (and evolution state of a lake), what is found in the upper sediment layer of a young lake should be found in the lower sediment layer of an older lake. In addition, the origin of spores will be investigated by comparing the lysis-resistant community found in upper sediment, with the communities (both total and lysis-resistant) from the overlying water column, surrounding soils, and river inflows. Results of this study might provide precious data for the understanding of the dynamic of spore-forming community in the environment and bring new insights into the ecological significance of the "seed bank" component of the bacterial community.

7.3.4 Natural background of ARG in the environment

The environmental impact of intensive antibiotic usage on the spread and accumulation of both antibiotics and their associated resistance determinants have received increasing attention these past few years. However, most of the studies concern highly human-impacted environments, like WWTP outlets or agriculture/farming soils (see introduction). There are relatively few data about the ARG levels in natural environments, with no or little impact by human activities. Additional data on the environmental background of ARG would help to evaluate the impact of intensive use of antibiotics and release of ARG-bearing organisms. Such knowledge would also be very useful for the implementation of new regulations and policies on the discharge standards.

Using a selection of samples from our own collection, we aim to provide a broader vision of the ARG distribution and levels across various environments, from different geographical location and differently affected by human activities. This selection includes samples from Antarctica, Botswana (Lake Liambezi and Okavango Delta), Swiss alpine lakes (pristine or bordered by grazing land), which will complement the data obtained in Lake Geneva.

7.3.5 Screening of ARG from all classes in spore-formers

Our findings provide the first evidence of ARG detection in spores or spore-like structures, demonstrating the applicability to use spore DNA as a proxy for tracking ARG in the environment for two selected genes. However, ARG are very diverse and include resistance to almost all classes of antibiotics known (Lee Ventola, 2015). Giving the apparent heterogeneous distribution of ARG among taxa, a systematic evaluation of other genes is necessary to determine the best applicability of our method. Depending on local usage policies (quantity, period), the frequency of each specific ARG within the spore-formers, and the efficiency of the detection/quantification protocol, the relevance of the choice of specific genes might be evaluated case-by-case, depending on the study context.

7.3.6 Cross-analysis of genes and genomes from human microbiome

The results of our studies suggest a heterogeneous distribution ARG among bacterial taxa. This assumption is mainly based on the results from the correlation tests, which have to be interpreted

with care, particularly in the case of the Vidy Bay. Due to the high impact of the WWTP, phenomena of co-deposition might shape the spatial distribution pattern of the community. Since it is likely that a part of the community (if not the main part) is inactive at time of deposition, the community structure can be assumed to reflect the composition of the deposited material, rather than the environmental conditions. Extreme deposition events (flooding events, release of untreated waters) might result in correlations between bacterial taxa solely based on their co-occurrence in the deposited material. If one of these taxa correlates with one ARG, the other taxa could also correlate, but without a real sense of causality. The same could be the case in the sediments of the Rhône Delta, but the various possible origins of lysis-resistant structures coupled to the variability of community over time should attenuate the covariance of these populations. Indeed, it is less likely that deposition of different taxa follows the same deposition pattern over time.

Although our studies highlighted correlations between ARG and certain specific taxa (genera), and gives potential candidates for ARG carriers, these correlation tests do not prove the presence of ARG in a taxon. This illustrates the limit of the metagenomic approach used in these studies. To further investigate the distribution of ARG and undoubtedly establish the presence of an ARG in specific taxa, alternative approaches have to be used. A following step is to try to identify the resistance genes directly in the genome of the human microbiota. For that, a cross-analysis of a wide variety of ARG and a collection of complete genomes from the human microbiota is in progress. Likewise, the same analysis will be performed for the screening of genes involved in the sporulation. The results of such analyses will provide evidence for the presence/ absence of ARG in specific taxa, and an overview of their taxonomic distribution. It will also increase knowledge about the diversity of spore-former, by potentially identifying sporulation determinants in “new” species or taxonomic groups.

7.3.7 Evaluate the effect of wastewater treatment on the spore-formers community

Wastewater treatment plants (WWTP) have been identified as hotspots for the dissemination of antibiotic resistant bacteria (ARBs) and ARG (see introduction). Although the impact of WWTP and their effluents is well reported, selection processes and the effect of different treatment types on the diversity and abundance of ARG in the WWTP are still not well understood, and studies are contradictory. Although a general decrease of ARG abundance is generally reported (Munir, Wong & Xagoraraki, 2011; Czekalski et al., 2012; Laht et al., 2014), frequency is commonly reported to increase, at least for some ARG (Bouki, Venieri & Diamadopoulos, 2013; Michael et al., 2013; Mao et al., 2015; Rodriguez-Mozaz et al., 2015), suggesting selection processes. WWTP are also proposed to be a reservoir of resistance, where environmental conditions would enhance gene transfer (Berendonk et al., 2015; Karkman et al., 2018).

In that context, the survival ability of human-associated spore-formers, and the factors controlling sporulation before and during wastewater treatment is of particular importance. This will define their potential to interact and exchange genes among themselves and with endogenous community. Currently it is not clear where sporulation occurs (in the human gut, before entering the WWTP, during treatment, in the environment). Inactive spores might not be able to interact with other populations, and acquire resistance. Moreover, we can assume that active human-associated and lysis-resistant structures such as spores will not require the same treatment to be removed, or at least reduced. Therefore, a screening of the cellular state of the human-associated microbiota (vegetative vs spore)

at the different steps of the wastewater treatment, by comparing total and lysis-resistant communities, might offer new insights on sporulation dynamics. The efficiency of wastewater treatment is of main importance if one wants to limit antibiotics and ARG pollution. Hence, a better understanding and control of the decontamination processes, based on the origin and the content of wastewaters, is essential for the risk assessment and might help to define adapted treatment.

Likewise, sediments and biofilms are also considered as hotspots of bacterial activity, where HGT rate is enhanced (see above). These hotspots represent environments where pathogens, human-associated and environmental organisms can meet and exchange genes, feeding the environmental reservoir of ARG and possibly transmit in return to pathogens and human-associated organisms. More attention must be paid on the key compartments, where bacterial density, diversity and activity is high, favoring interactions and gene transfer. Investigating the presence of lysis-resistant organisms associated to the human microbiota in these compartments will provide precious information for a better understanding of the potential ARG dissemination mediated by this resilient community.

7.3.8 In vivo experiments

Finally, one important question that we would like to address is how the exposure to antibiotics affects the shape of microbiome resistome, and more specifically in the spore-forming fraction of the community. On the one hand, selection pressure due to antibiotic stress increases ARG frequency. Indeed, surviving populations have higher frequency of ARG, inducing higher chance to share them. On the other hand, antibiotic stress is also supposed to stimulate HGT. Although this latter is commonly accepted, there is a lack of data demonstrating this phenomenon (Lopatkin, Sysoeva & You, 2016). Organisms able to enter a dormant state are thought to have more chance to acquire a beneficial mutations, because they survive longer (Levin-Reisman et al., 2017). We can then assume that they also increase their chance to acquire a gene by HGT. However, currently there is no data proving that theory. Altogether, these considerations suggest that spore-forming bacteria have a predisposition for the acquisition of ARG under antibiotic exposure.

Here we propose to conduct an *in vivo* experiment on model organisms (mice) for testing the effect of antibiotics on: (i) the composition of both the total and the lysis-resistant communities, (ii) the frequency of ARG associated to these communities, (iii) and the spread of resistance within and across taxa. Composition of both the total and the spore communities will be analyzed on faeces from these model organisms, and a follow-up of the evolution of the microbiome exposed to different classes and concentrations of antibiotics will be performed. Whole genome sequencing would also allow detecting the appearance of resistance in formerly non-resistant taxa. Results of such experiments would greatly increase our knowledge of the dynamics of lysis-resistant bacteria populations and its reactions to antibiotic exposure, and help to clarify the presumable ability of spore-formers to acquire ARG.

The detection of ARG in environmental samples and their attribution to specific organisms is challenging, due to methodological considerations. New technologies, such as new high-throughput single-cell genome sequencing (Lan et al., 2017) or epicPCR techniques (Spencer et al., 2016), will provide powerful tools to overcome these limitations. This could be of particular interest to study the distribution of ARG among taxa, and the HGT rate among and across taxa, notably in the case of the WWTP or the in vivo experiments.

A better understanding of the role and fate of the spore-formers and other lysis-resistant organisms in the environment, their interactions with environmental strains, as well as a screening of ARG in the spore-forming or lysis-resistant populations might help to develop appropriate strategies for the usage management of antibiotics, and for the efficient treatment of contaminated wastewater.

7.4 References

Aminov RI. 2010. A brief history of the antibiotic era: Lessons learned and challenges for the future. *Frontiers in Microbiology* 1:1–7. DOI: 10.3389/fmicb.2010.00134.

Bartholomew JW., Paik G. 1966. Isolation and identification of obligate thermophilic sporeforming bacilli from ocean basin cores. *Journal of Bacteriology* 92:635–638.

Barton L. 2005. *Structural and Functional Relationships in Prokaryotes*. Springer Science & Business Media.

Bengtsson-Palme J., Kristiansson E., Larsson DGJ. 2017. Environmental factors influencing the development and spread of antibiotic resistance. *FEMS Microbiology Reviews*:1–13. DOI: 10.1093/femsre/fux053.

Berendonk TU., Manaia CM., Merlin C., Fatta-Kassinos D., Cytryn E., Walsh F., Bürgmann H., Sørum H., Norström M., Pons MN., Kreuzinger N., Huovinen P., Stefani S., Schwartz T., Kisand V., Baquero F., Martinez JL. 2015. Tackling antibiotic resistance: The environmental framework. *Nature Reviews Microbiology* 13:310–317. DOI: 10.1038/nrmicro3439.

Boere AC., Sinninghe Damsté JS., Rijpstra WIC., Volkman JK., Coolen MJL. 2011. Source-specific variability in post-depositional DNA preservation with potential implications for DNA based paleoecological records. *Organic Geochemistry* 42:1216–1225. DOI: 10.1016/j.orggeochem.2011.08.005.

Bouki C., Venieri D., Diamadopoulos E. 2013. Detection and fate of antibiotic resistant bacteria in wastewater treatment plants: A review. *Ecotoxicology and Environmental Safety* 91:1–9. DOI: 10.1016/j.ecoenv.2013.01.016.

Browne HP., Forster SC., Anonye BO., Kumar N., Neville BA., Stares MD., Goulding D., Lawley TD. 2016. Culturing of 'unculturable' human microbiota reveals novel taxa and extensive sporulation. *Nature* 533:543–546. DOI: 10.1038/nature17645.

Bucci JP., Szempruch AJ., Caldwell JM., Ellis JC., Levine JF. 2014. Seasonal changes in microbial community structure in freshwater stream sediment in a North Carolina river basin. *Diversity* 6:18–32. DOI: 10.3390/d6010018.

Bueche M., Wunderlin T., Roussel-Delif L., Junier T., Sauvain L., Jeanneret N., Junier P. 2013. Quantification of endospore-forming firmicutes by quantitative PCR with the functional gene spo0A. *Applied and Environmental Microbiology* 79:5302–5312. DOI: 10.1128/AEM.01376-13.

Chen N., Yang JS., Qu JH., Li HF., Liu WJ., Li BZ., Wang ET., Yuan HL. 2015. Sediment prokaryote communities in different sites of eutrophic Lake Taihu and their interactions with environmental factors. *World Journal of Microbiology and Biotechnology* 31:883–896. DOI: 10.1007/s11274-015-1842-1.

Czekalski N., Berthold T., Caucci S., Egli A., Bürgmann H. 2012. Increased levels of multiresistant bacteria and resistance genes after wastewater treatment and their dissemination into Lake Geneva, Switzerland. *Frontiers in Microbiology* 3:106. DOI: 10.3389/fmicb.2012.00106.

Forster SC., Kumar N., Anonye BO., Almeida A., Viciani E., Stares MD., Dunn M., Mkandawire TT., Zhu A., Shao Y., Pike LJ., Louie T., Browne HP., Mitchell AL., Neville BA., Finn RD., Lawley TD. 2019. A human gut bacterial genome and culture collection for improved metagenomic analyses. *Nature Biotechnology* 37:186–192. DOI: 10.1038/s41587-018-0009-7.

Hinder B., Gabathuler M., Steiner B., Hanselmann K., Preisig HR. 1999. Seasonal dynamics and

phytoplankton diversity in high mountain lakes (Joeri lakes, Swiss Alps). *Journal of Limnology* 58:152–161.

Hullar MAJ., Kaplan LA., Stahl DA. 2006. Recurring seasonal dynamics of microbial communities in stream habitats. *Applied and Environmental Microbiology* 72:713–722. DOI: 10.1128/AEM.72.1.713-722.2006.

Junier T., Hervé V., Wunderlin T., Junier P. 2015. MLgsc: A maximum-likelihood general sequence classifier. *PLoS ONE* 10:1–12. DOI: 10.1371/journal.pone.0129384.

Karkman A., Do TT., Walsh F., Virta MPJ. 2018. Antibiotic-Resistance Genes in Waste Water. *Trends in Microbiology* 26:220–228. DOI: 10.1016/j.tim.2017.09.005.

Laht M., Karkman A., Voolaid V., Ritz C., Tenson T., Virta M., Kisand V. 2014. Abundances of tetracycline, sulphonamide and beta-lactam antibiotic resistance genes in conventional wastewater treatment plants (WWTPs) with different waste load. *PLoS ONE* 9:1–8. DOI: 10.1371/journal.pone.0103705.

Lan F., Demaree B., Ahmed N., Abate AR. 2017. Single-cell genome sequencing at ultra-high-throughput with microfluidic droplet barcoding. *Nature Biotechnology* 35:640–646. DOI: 10.1038/nbt.3880.

Lee Ventola C. 2015. The antibiotic resistance crisis: Part 1: causes and threats. *P & T journal* 40:277–83. DOI: Article.

Lennon JT., Jones SE. 2011. Microbial seed banks: the ecological and evolutionary implications of dormancy. *Nature Reviews Microbiology* 9:119–130. DOI: 10.1038/nrmicro2504.

Levin-Reisman I., Ronin I., Gefen O., Braniss I., Shoresh N., Balaban NQ. 2017. Antibiotic tolerance facilitates the evolution of resistance. *Science* 355:826–830. DOI: 10.1126/science.aaj2191.

Lopatkin AJ., Sysoeva TA., You L. 2016. Dissecting the effects of antibiotics on horizontal gene transfer: Analysis suggests a critical role of selection dynamics. *BioEssays* 38:1283–1292. DOI: 10.1002/bies.201600133.

Mao D., Yu S., Rysz M., Luo Y., Yang F., Li F., Hou J., Mu Q., Alvarez PJJ. 2015. Prevalence and proliferation of antibiotic resistance genes in two municipal wastewater treatment plants. *Water Research* 85:458–466. DOI: 10.1016/j.watres.2015.09.010.

Marti E., Variatza E., Balcazar JL. 2014. The role of aquatic ecosystems as reservoirs of antibiotic resistance. *Trends in Microbiology* 22:36–41. DOI: 10.1016/j.tim.2013.11.001.

Michael I., Rizzo L., McArdell CS., Manaia CM., Merlin C., Schwartz T., Dagot C., Fatta-Kassinos D. 2013. Urban wastewater treatment plants as hotspots for the release of antibiotics in the environment: A review. *Water Research* 47:957–995. DOI: 10.1016/j.watres.2012.11.027.

Mukhopadhya I., Moraïs S., Laverde-Gomez J., Sheridan PO., Walker AW., Kelly W., Klieve A V., Ouwerkerk D., Duncan SH., Louis P., Koropatkin N., Cockburn D., Kibler R., Cooper PJ., Sandoval C., Crost E., Juge N., Bayer EA., Flint HJ. 2018. Sporulation capability and amylosome conservation among diverse human colonic and rumen isolates of the keystone starch-degrader Ruminococcus bromii. *Environmental Microbiology* 20:324–336. DOI: 10.1111/1462-2920.14000.

Munir M., Wong K., Xagoraraki I. 2011. Release of antibiotic resistant bacteria and genes in the effluent and biosolids of five wastewater utilities in Michigan. *Water Research* 45:681–693. DOI: 10.1016/j.watres.2010.08.033.

Nicholson WL. 2002. Roles of Bacillus endospores in the environment. *Cellular and Molecular Life Sciences* 59:410–416. DOI: 10.1007/s00018-002-8433-7.

Nicholson WL., Munakata N., Horneck G., Melosh HJ., Setlow P. 2000. Resistance of Bacillus endospores to extreme terrestrial and extraterrestrial environments. *Microbiology and molecular biology reviews* 64:548–72. DOI: 10.1128/MMBR.64.3.548-572.2000.

Pruden A., Pei R., Storteboom H., Carlson KH. 2006. Antibiotic Resistance Genes as Emerging Contaminants: Studies in Northern Colorado †. *Environmental Science & Technology* 40:7445–7450. DOI: 10.1021/es060413l.

de Rezende JR., Kjeldsen KU., Hubert CRJ., Finster K., Loy A., Jørgensen BB. 2013. Dispersal of thermophilic Desulfotomaculum endospores into Baltic Sea sediments over thousands of years. *The ISME Journal* 7:72–84. DOI: 10.1038/ismej.2012.83.

Rodriguez-Mozaz S., Chamorro S., Marti E., Huerta B., Gros M., Sànchez-Melsió A., Borrego CM., Barceló D., Balcázar JL. 2015. Occurrence of antibiotics and antibiotic resistance genes in hospital and urban wastewaters and their impact on the receiving river. *Water research* 69:234–242. DOI: 10.1016/j.watres.2014.11.021.

Rothfuss F., Bender M., Conrad R. 1997. Survival and activity of bacteria in a deep, aged lake sediment (Lake Constance). *Microbial Ecology* 33:69–77. DOI: 10.1007/s002489900009.

van Schaik W. 2015. The human gut resistome. *Philosophical Transactions of the Royal Society B: Biological Sciences* 370:20140087–20140087. DOI: v.

Shoemaker WR., Lennon JT. 2018. Evolution with a seed bank: The population genetic consequences of microbial dormancy. *Evolutionary Applications* 11:60–75. DOI: 10.1111/eva.12557.

Spencer SJ., Tamminen M V., Preheim SP., Guo MT., Briggs AW., Brito IL., A Weitz D., Pitkänen LK., Vigneault F., Virta MP., Alm EJ. 2016. Massively parallel sequencing of single cells by epicPCR links functional genes with phylogenetic markers. *ISME Journal* 10:427–436. DOI: 10.1038/ismej.2015.124.

Taylor NGH., Verner-Jeffreys DW., Baker-Austin C. 2011. Aquatic systems: Maintaining, mixing and mobilising antimicrobial resistance? *Trends in Ecology and Evolution* 26:278–284. DOI: 10.1016/j.tree.2011.03.004.

Tetz G., Tetz V. 2017. Introducing the sporobiota and sporobiome. *Gut Pathogens* 9:1–6. DOI: 10.1186/s13099-017-0187-8.

Tocheva EI., Ortega DR., Jensen GJ. 2016. Sporulation, bacterial cell envelopes and the origin of life. *Nature Reviews Microbiology* 14:535–542. DOI: 10.1038/nrmicro.2016.85.

de Vries LE., Vallès Y., Agersø Y., Vaishampayan PA., García-Montaner A., Kuehl J V., Christensen H., Barlow M., Francino MP. 2011. The gut as reservoir of antibiotic resistance: Microbial diversity of tetracycline resistance in mother and infant. *PLoS ONE* 6. DOI: 10.1371/journal.pone.0021644.

Wunderlin T., Corella JP., Junier T., Bueche M., Loizeau J-L., Girardclos S., Junier P. 2014a. Endospore-forming bacteria as new proxies to assess impact of eutrophication in Lake Geneva (Switzerland-France). *Aquatic Sciences* 76:103–116. DOI: 10.1007/s00027-013-0329-0.

Wunderlin T., Junier T., Paul C., Jeanneret N., Junier P. 2016. Physical Isolation of Endospores from Environmental Samples by Targeted Lysis of Vegetative Cells. *JOVE-Journal of Visualized Experiments*. DOI: 10.3791/53411.

Wunderlin T., Junier T., Roussel-Delif L., Jeanneret N., Junier P. 2013. Stage 0 sporulation gene A as a molecular marker to study diversity of endospore-forming Firmicutes. *Environmental Microbiology Reports* 5:911–924. DOI: 10.1111/1758-2229.12094.

Wunderlin T., Junier T., Roussel-Delif L., Jeanneret N., Junier P. 2014b. Endospore-enriched sequencing

approach reveals unprecedented diversity of Firmicutes in sediments. *Environmental Microbiology Reports* 6:631–639. DOI: 10.1111/1758-2229.12179.

Yuhana M., Horath T., Hanselmann K. 2006. Bacterial community shifts of a high mountain lake in response to variable simulated conditions availability of nutrients, light and oxygen. *Biotropia* 13:85–98.

Zhang L., Zhao T., Shen T., Gao G. 2019. Seasonal and spatial variation in the sediment bacterial community and diversity of Lake Bosten, China. *Journal of Basic Microbiology* 59:224–233. DOI: 10.1002/jobm.201800452.

8 Other projects and collaborations

Foreword

In this chapter, I present a few projects in which I was also involved during my PhD.

8.1 Physical Isolation of Endospores from Environmental Samples by Targeted Lysis of Vegetative Cells

Tina Wunderlin, Thomas Junier, Christophe Paul, Nicole Jeanneret, Pilar Junier

2016, Journal of Visualized Experiments (Issue 107, pp. e53411), DOI: 10.3791/53411

Foreword

This chapter is a video article showing how to realize the isolation of endospores, a method developed by Tina Wunderlin and previously described in "Endospore-enriched sequencing approach reveals unprecedented diversity of Firmicutes in sediments" (Environmental Microbiology Reports, Vol. 6, Issue 6, pp. 631-639, 2016).

My contribution to this publication included some modifications of the original protocols, to allow the simultaneous treatment of multiple samples, the preparation of the experiment, before and during the recording, as well as a participation to the written part accompanying the video.

jove Journal of Visualized Experiments www.jove.com

Video Article

Physical Isolation of Endospores from Environmental Samples by Targeted Lysis of Vegetative Cells

Tina Wunderlin[1], Thomas Junier[2], Christophe Paul[3], Nicole Jeanneret[3], Pilar Junier[3]

[1]Department of Biological Sciences, Macquarie University

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

[3]Laboratory of Microbiology, University of Neuchatel

Correspondence to: Pilar Junier at pilar.junier@unine.ch

URL: http://www.jove.com/video/53411
DOI: doi:10.3791/53411

Keywords: Environmental Sciences, Issue 107, Endospores, separation, vegetative cells, endospore-forming bacteria, cell lysis, diversity, microbial ecology

Date Published: 1/21/2016

Citation: Wunderlin, T., Junier, T., Paul, C., Jeanneret, N., Junier, P. Physical Isolation of Endospores from Environmental Samples by Targeted Lysis of Vegetative Cells. *J. Vis. Exp.* (107), e53411, doi:10.3791/53411 (2016).

Abstract

Endospore formation is a survival strategy found among some bacteria from the phylum Firmicutes. During endospore formation, these bacteria enter a morpho-physiological resting state that enhances survival under adverse environmental conditions. Even though endospore-forming Firmicutes are one of the most frequently enriched and isolated bacterial groups in culturing studies, they are often absent from diversity studies based on molecular methods. The resistance of the spore core is considered one of the factors limiting the recovery of DNA from endospores. We developed a method that takes advantage of the higher resistance of endospores to separate them from other cells in a complex microbial community using physical, enzymatic and chemical lysis methods. The endospore-only preparation thus obtained can be used for re-culturing or to perform downstream analysis such as tailored DNA extraction optimized for endospores and subsequent DNA sequencing. This method, applied to sediment samples, has allowed the enrichment of endospores and after sequencing, has revealed a large diversity of endospore-formers in freshwater lake sediments. We expect that the application of this method to other samples will yield a similar outcome.

Video Link

The video component of this article can be found at http://www.jove.com/video/53411/

Introduction

The goal of this work is to provide a protocol for the separation of bacterial endospores from vegetative bacterial cells in environmental samples. The formation of bacterial endospores is a survival strategy, usually triggered by starvation, found in a number of bacterial groups belonging to the phylum Firmicutes[1]. Endospore-forming bacteria are well studied, mainly because a number of strains are pathogens and hence of medical importance (*e.g., Bacillus anthracis* or *Clostridium difficile*). Environmental strains of endospore-forming bacteria have been isolated from virtually every environment (soil, water, sediment, air, ice, human gut, animals gut, and more)[1-3]. Therefore, Firmicutes are the second most abundant phylum in culture collections[4].

Because of their hardy outer cortex and protective core proteins, endospores can survive extreme environmental conditions ranging from desiccation to high radiation, extreme temperatures and harmful chemicals[5]. This remarkable resistance makes it a challenge to extract DNA from endospores[6-8]. This likely explains why they have been overlooked in environmental sequencing studies[9,10]. Other methods, such as targeting of endospores in environmental samples by fluorescent antibodies[11], quantification of dipicolinic acid (DPA) in soil[12] and sediment[13], flow cytometry[14] or pasteurization and subsequent cultivation[15,16] have been used to retrieve or quantify endospores in environmental samples. In recent years, optimized DNA extraction methods as well as specific molecular primers to target endospore-specific gene sequences have been developed[10,17-20]. This has helped to reveal more biodiversity among this group of bacteria[21] and has also led to applications in industry and medicine for the detection of endospores, for example in milk powder[19].

The protocol presented here is based on the difference in resistance to harmful physicochemical conditions (such as heat and detergents) of bacterial endospores relative to vegetative cells. To destroy vegetative cells in a sample, we consecutively apply heat, lysozyme and low concentrations of detergents. The time and strength of these treatments have been optimized so as not to destroy spores, but to lyse all vegetative cells. Some cells in an environmental cell pool are more resistant than others, so in order to increase the probability of destroying all vegetative cells, we apply three different treatments. The advantage and novelty of this method is that the endospores after the treatment are still intact and can be used for further downstream analyses. These include DNA extraction, quantitative PCR (qPCR) and amplicon or metagenomic sequencing (targeting specifically the group of endospores and thus reducing diversity, while increasing coverage). The endospores could also be used for downstream cultivation or quantification by fluorescence microscopy, flow cytometry, or detection of DPA. An important feature of this method is that by comparing an untreated sample with a treated sample, one can deduce the quantity and diversity of endospores in an environmental sample in addition to the component corresponding to vegetative cells.

jove Journal of Visualized Experiments www.jove.com

Protocol

1. Preparation of Chemicals and Equipment

1. Make 500 ml of a 1% sodium hexametaphosphate (SHMP) $(NaPO_3)_n$ solution and sterilize by autoclaving.
2. Sterilize nitrocellulose (NC) filters (pore size 0.22 µm, diameter 47 mm) by autoclaving in closed glass Petri dishes.
3. Sterilize NC filters (pore size 0.22 µm, diameter 25 mm) by autoclaving in closed glass Petri dishes.
4. Weigh and note the empty weight of sterile 50 ml tubes (with cap on) (one tube per sample).
5. Prepare Tris-EDTA-buffer (TE-buffer) 1x: make solution of 10 mM Tris (*tris*(hydroxymethyl)aminomethane) and 1 mM EDTA buffer. Adjust pH to 8 and sterilize by autoclaving.
6. Prepare lysozyme solution (20 mg/ml) by dissolving 0.02 g of lysozyme in 1 ml TE-buffer. Ideally, make fresh every time. Store at 4 °C for no more than 1 week.
7. Prepare physiological solution by dissolving 8 g/L sodium chloride (NaCl) in distilled water. Sterilize by autoclaving.

2. Separation of Biomass from Sediment

1. Add 3 g of sediment sample to pre-weighed sterile 50 ml tubes using ethanol-flamed metal scoops. Perform this in a clean, UV sterilized biosafety cabinet to avoid contamination.
2. Add 15 ml of a 1% sterile (autoclaved) SHMP solution to the sample using a sterile graduated burette. The SHMP solution can also be filtered to avoid contamination.
3. Homogenize the sediment and SHMP solution with a liquid disperser/homogenizer (*e.g.,* Ultra-Turrax homogenizer). 70% ethanol-sterilize or autoclave the dispersion rotor prior to use. Run the homogenization for 1 min at 17,500 rpm. Let the sample rest for 2 min and repeat the homogenization for 1 min at the same rotor speed.
4. Let the sample stand for 10 min. At this step, the heaviest particles (minerals) will settle. The cells and any organic components of the sample however will remain in solution. Afterwards, transfer the supernatant solution (containing cell biomass) into a clean 50 ml tube, while taking care not to disturb the sediment pellet.
5. To the sediment pellet add again 15 ml of a 1% sterile (autoclaved) SHMP solution using a sterile graduated burette. Then repeat steps 2.3 and 2.4. This repetition ensures the separation of the maximum amount of cells and organic particles from the mineral component of the sediment. The supernatant of this second separation can be merged with the supernatant from the first separation.
   Note: The following steps are all done on the supernatant (containing cell biomass). The mineral component (sediment pellet) can be discarded.
6. Centrifuge the sample at 20 x g for 1 min. This step increases the g-force enough to settle small mineral particles while the biological cell material still remains in solution. After centrifugation, transfer the supernatant solution (containing cell biomass) into a clean 50 ml tube. Discard the mineral pellet.
7. Determine the final volume of the solution containing biomass by weighing the sample. The weight determination avoids having to transfer the sample to a graduated cylinder and reduces risk of contamination.

3. Collection of Biomass on Filter Membrane

1. Prepare filtration unit (for 47 mm diameter membranes) and vacuum pump. Sterilize the filtration unit either by autoclaving or (if Pyrex glass) by spraying it with 70% ethanol and flaming it with a Bunsen burner. Let it cool down before continuing with the protocol.
2. Add sterile NC membrane to the filtration unit using ethanol-flamed sterilized forceps.
3. Add half of the supernatant sample (from step 2.6) onto the membrane filtration unit and collect cells on the membrane using the vacuum pump.
4. When the liquid has fully passed through the filter, stop the vacuum pump and carefully remove the membrane using ethanol-flame sterilized forceps. Place the membrane into a sterile Petri dish.
    1. Cut the membrane in half using ethanol-flamed sterilized scissors. Add each half of the membrane to a separate 2 ml tube. One half of the membrane will be used for DNA extraction and analysis of the entire bacterial community. The other half of filter will be stored at -80 °C and serves as a backup.
5. Place new NC membrane onto the filtration unit and collect biomass from the second half of sample volume (from step 2.6) using the vacuum pump.
6. When the liquid has fully passed through the filter, stop the vacuum pump and carefully remove the membrane using ethanol-flamed sterilized forceps. Place the entire membrane into a separate 2 ml tube. This sample will be used for the treatment to separate endospores from vegetative cells. The sample can be stored at -20 °C until use.

4. Lysis of Vegetative Cells

1. Perform the treatment to separate endospores from vegetative cells on the biomass previously collected on a NC membrane (step 3.6).
    1. If the membrane was frozen, leave it at RT for 10 min to thaw. Then place the membrane in a sterile Petri dish and cut it (approximately 4 times) into smaller pieces using ethanol-flamed sterilized scissors. Then place all membrane filter pieces into a sterile 2 ml tube.
2. Add 900 µl of 1x TE (Tris-EDTA) buffer (see 1.5) to the tube containing the sample membrane and mix thoroughly by vortex. At this step, the biomass is removed from the membrane into the TE-buffer solution.
3. Place the tube in an incubator at 65 °C for 10 min and 80 rpm. Afterwards remove the tube from the incubator and let it cool down for 15 min.

jove Journal of Visualized Experiments www.jove.com

4. Add 100 µl of freshly prepared lysozyme (see 1.5) to reach a final concentration of 2 mg/ml. Do not add the lysozyme before the sample has cooled down to 37 °C, as this could degrade the enzyme.
5. Incubate the sample at 37 °C for 60 min and 80 rpm, the optimal conditions for the lysozyme to lyse vegetative cells.
6. After lysis is complete, add 250 µl of 3 N sodium hydroxide (NaOH) and 250 µl of 6% sodium dodecyl sulfate (SDS) solution to the sample. By adding this, the sample volume reaches 1.5 ml and there is a final concentration of 0.5 N NaOH and final concentration of 1% SDS.
7. Incubate this mix at RT for 60 min and 80 rpm. Adding the base and detergents will help in final cell lysis. The concentration of these detergents has been optimized as to not harm the endospores, while lysing vegetative cells.
8. Prepare a sterile filtration unit that holds 25 mm diameter membranes by autoclaving, or (if Pyrex glass) spraying it with 70% ethanol and flaming it with a Bunsen burner. Let it cool down.
9. Place a 0.2 µm NC membrane (25 mm diameter) on the filtration unit using ethanol-flame sterilized forceps.
10. Add the sample from step 4.7 onto the membrane and filter the liquid using the vacuum pump. When liquid has passed through, turn off vacuum pump. At this step, the lysed vegetative cell material is removed, as it is not retained on the membrane. Only endospores will remain on the membrane.
11. Add 2 ml of sterile physiological solution to wash off residual detergents and filter the liquid using the vacuum pump.
12. When liquid has fully filtered, turn off the vacuum pump. Leave the membrane on the filtration unit.

5. DNase Treatment

Note: Perform the DNase treatment directly on the filter membrane. It is important that the filtration unit does not leak and the vacuum pump is turned off.

1. Add 450 µl of sterile water, 50 µl of DNase reaction buffer (1x) and 0.5 µl DNase enzyme directly onto the filter membrane and let it stand for 15 min. If possible, do this digestion in a room that is slightly warmer than average RT, since the enzyme works better at temperatures of 25 °C and above.
   Note: Keeping the Bunsen burner aside the filtration unit also increases the temperature and has the added benefit of keeping the surroundings sterile, therefore reduced risk of contamination of the samples.
2. When the DNase digestion is finished, turn on vacuum pump to remove the enzyme from the sample.
3. Wash off residual enzyme by adding and filtering 1 ml of physiological solution.
4. If liquid has fully passed, turn off vacuum pump and remove the filter membrane containing endospores using sterile forceps and place it into a sterile Petri dish.
   Note: The sample of separated endospores is now ready for downstream analysis. Store at -20 °C if used for DNA extraction or alternatively at 4°C if used for germination and cultivation.

Representative Results

The results presented here have been published earlier[10,21]. Please refer to those articles for the environmental interpretation and discussion of the data.

The overall procedure is summarized in **Figure 1** and corresponds to three main steps: first, the separation of biomass from sediment or any other environmental matrix; second, the destruction of vegetative cells; and third, the downstream analysis of the separated endospores. Downstream analysis could consist, for example, of DNA extraction and amplicon sequencing to determine diversity. The DNA extraction needs to be optimized as to guarantee lysis of endospores. In sediment samples, we have achieved this by using a forceful sequential DNA extraction procedure[10]. The application of the method in sediment demonstrates a significant increase in the fraction of endospore-forming Firmicutes after separation. In amplicon sequencing of the 16S rRNA gene, Firmicutes in the untreated samples (whole community) corresponded only to 8.0 and 19.0% of the sequences (**Table 1**). In contrast, after the treatment Firmicutes represented 90.6% and 83.9% of the endospore-enriched sample. The two major orders of endospore-forming Firmicutes, Bacilliales and Clostridiales, were enriched with the method, contrasting with the absence of orders like Lactobacilliales that are non endospore-forming Firmicutes. In order to demonstrate that the method is particularly suitable for the enrichment of true endospores, other groups capable of producing spore-like structures were also analyzed. Accordingly, the frequency of Actinobacteria, Cyanobacteria and Myxococcales decreased after the treatment to lyse cells.

The efficiency of the treatment was also demonstrated using pure cultures. An endospore preparation (> 95% endospores) of *Paenibacillus alvei, Bacillus subtilis* and *Bacillus megaterium*, as well as a vegetative cell culture of *Escherichia coli,* were treated as described in the protocol for the lysis of vegetative cells. All cultures were then incubated in nutrient broth at 37 °C and growth was measured using optical density at 600 nm wavelength. Growth was observed for the endospore cultures while no growth was observed in the treated *E. coli* culture, proving that vegetative cells are irreversibly damaged (**Figure 2**).

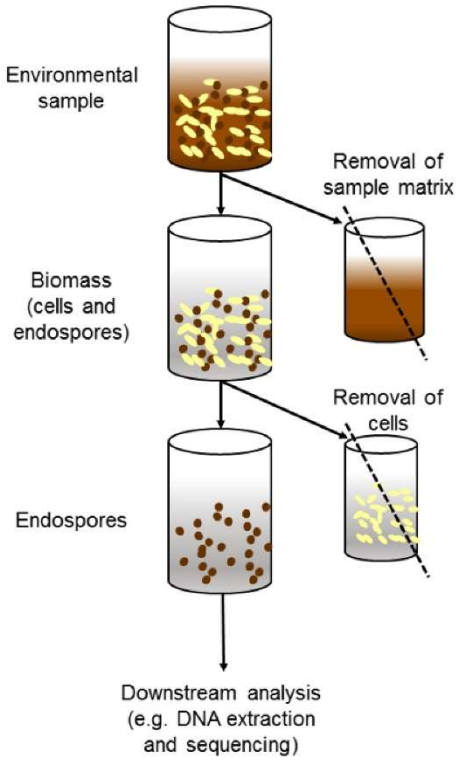


Figure 1. Overview of the experimental procedure. Procedure used to enrich endospore-forming bacteria in environmental samples. The step of separating cells and endospores from the environmental matrix can be omitted depending on the type of sample (*i.e.*, water sample). In the figure the downstream methods applied on the spore-enriched fraction correspond to DNA extraction and high throughput sequencing. These steps can be replaced by culturing. Please click here to view a larger version of this figure.

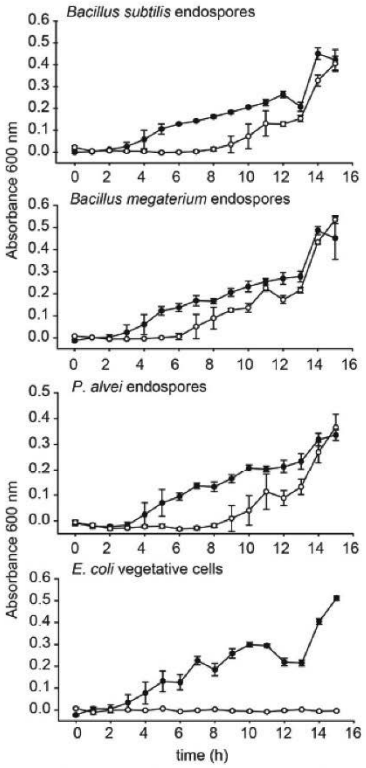


Figure 2. Verification of the treatment on pure cultures. Growth curves verifying the growth of endospore-forming bacteria from a suspension of endospores of *Bacillus subtilis, Bacillus megaterium,* and *Paenibacillus alvei* and a cell culture of *Escherichia coli* treated with the method for the lysis of cells. Treated = ○. Untreated = ●. Error bars from three independent cultures. Growth of control cultures and re-growth of treated cultures was measured as optical density at 600 nm wavelength. This figure has been re-printed from Wunderlin *et al.* 2014 with permission. Please click here to view a larger version of this figure.

	Sediment 1		Sediment 2	
	whole community	**endospore-enriched**	**whole community**	**endospore-enriched**
Firmicutes	8.0	90.6	19.0	83.9
Bacilli	0.5	10.0	5.7	15.1
Clostridia	7.4	76.9	12.5	63.2
Actinobacteria	2.4	1.0	4.4	2.1
Cyanobacteria	1.1	0.1	0.7	0.1
Myxococcales	0.7	0.0	0.2	0.0
Other bacteria	87.8	8.3	75.7	13.9

Table 1. Abundance of endospores and other spore-forming bacterial groups. Relative frequency of Firmicutes (endospore-formers) and other bacterial groups producing spore-like structures in two sediment samples corresponding to the whole (untreated) and endospore-enriched (treated) communities.

Discussion

The resistance of endospores to external aggressive physicochemical factors (*e.g.,* temperature or detergents) was used to devise a method to separate bacterial endospores from vegetative cells in environmental samples. This is the first comprehensive method to isolate endospores from environmental samples in a non-destructive manner. Previous methods to quantify, detect or analyze endospores in samples were based on the measurement of specific proxies for endospores such as dipicolinic acid or specific marker genes. In contrast, with the protocol presented here, sample components other than endospores are removed and so the endospores eventually remain as the sole and purified remnants of the sample. Although other methods such as pasteurization or density gradient centrifugation have been proposed to enrich for endospores[22], our tests showed that density gradient centrifugation is not appropriate as a general method for environmental samples, due to physiological

differences and different densities of various species of endospore-formers (data not shown). By contrast, our protocol can easily be applied to all types of environmental samples and is suitable for downstream applications including molecular methods or culturing.

There are two critical steps in the protocol. The first is the separation of biomass from sample matrix (*i.e.,* sediment particles). It is essential that the number of cells separated from the matrix be maximized, so as not to overlook part of the diversity. For sediment samples, as used here, one of the principal limitations of the protocol is the fact that some cells or spores may not have been detached from the sediment matrix and therefore not included in the downstream analysis, which is a potential limitation of the method. Depending on the sample matrix (for example if there are humic acids), cells may be tightly bound to it and therefore difficult to release. The use of ortho-phosphate buffer as well as good homogenization with a homogenizer or blender are important at this step. To boost recovery, the procedure of homogenization and removal of biomass is repeated twice as written in the protocol. The protocol described here has been developed and optimized for samples of freshwater lake sediment. Some parameters may not be optimal for other types of samples. One way to analyze the community fraction that was potentially overlooked in the process would be to subject the sediment matrix to DNA extraction and amplicon sequencing. As possible modifications to the technique, the step of separation of biomass from sample matrix may be omitted. Particularly if the environmental sample does not contain organic material or mineral particles that could interfere with the downstream protocol (*e.g.,* water, other liquids or purified cultures), prior separation may not be needed. For liquid samples the protocol can start directly at chapter 3 (Collection of biomass on filter membrane).

The second important step of the protocol is the treatment with lysozyme, heat, NaOH and SDS to destroy vegetative cells. Temperature, duration as well as concentration of chemicals have all been optimized as not to harm endospores, while destroying the cells. These parameters should therefore be strictly kept as they are. The use of a first step of heating the sample at 65 °C was very effective to specifically select for endospores compared to other types of bacterial spores or spore-like structures occurring among the bacterial phyla of Actinobacteria, Myxobacteria, Cyanobacteria, that are generally not heat-resistant. As seen in **Table 1**, non-spore forming bacteria are still present after the treatment (8.3% to 14%). Some explanations for this are that sediment samples are highly complex and can also harbor resistant non-spore cells. In addition, leftover organic material may allow certain attached cells to survive the treatment. In order to further reduce the percentage of non-spore-forming cells in the treated samples, the method should be optimized based on individual sample type.

The method represents a novel tool for the targeted study of endospore-formers in environmental samples. The treatment to destroy vegetative cells can be tested on pure cultures of cells and endospores and adapted to suit individual resistance of cells. The destruction of cells can be seen in the curves of treated cultures shown in **Figure 2**. The delayed growth of the treated cultures can be due to the time that endospores need to re-germinate and move into the exponential growth phase. Other possible explanations for this delay could also be a weakening of the endospore culture or lower cell numbers after treatment. If the separated endospores are not used for DNA extraction, the last step of DNase treatment can be omitted.

Future applications of this technique could be envisaged in industries where pathogenic endospores need to be detected. One example is in the food industry and clinical research, where the detection and analysis (for identification) of endospores, and its differentiation from vegetative cells (for example, from foods products such as milk or milk-based products) is vital. This might be particular relevant considering that many standard procedures consider direct DNA extraction from a sample using lytic enzymes that are not adapted to lyse endospores, which will thus be overlooked. With the method presented here, cells can be removed and subsequent analysis will provide answers to the presence or absence of endospores as opposed to cells. Other applications include the analysis of endospore communities in different environmental samples (for example ice or sediment cores) that are historical archives of environmental conditions. In many environments with relatively stable environmental conditions (for example lake sediment surface) endospore-forming Firmicutes can thrive in the form of vegetative cells. When conditions deteriorate (for example by nutrient depletion during sediment burial or a shift towards anaerobic conditions) the cells move into endospore state. Therefore, the retrieved endospore communities from sediment cores can reflect the environmental conditions at time of burial and thus be used as a paleoecological indicator of the conditions prior to burial. However, we are still gathering information to support this claim.

Disclosures

The authors have nothing to disclose.

Acknowledgements

The authors acknowledge the Swiss National Science Foundation for Grant No. 31003A-132358/1, 31003A_152972 and No. 151948, and Fundation Pierre Mercier pour la Science.

References

1. Nicholson, W.L. Roles of Bacillus endospores in the environment. *Cell Mol Life Sci.* **59(3)**, 410-416 (2002).
2. Lester, E.D., Satomi, M., Ponce, A. Microflora of extreme arid Atacama Desert soils. *Soil Biol Biochem.* **39(2)**, 704-708 (2007).
3. Wilson, M.S., Siering, P.L., White, C.L., Hauser, M.E., & Bartles, A.N. Novel archaea and bacteria dominate stable microbial communities in North America's Largest Hot Spring. *Microb Ecol.* **56(2)**, 292-305 (2008).
4. Hugenholtz, P. Exploring prokaryotic diversity in the genomic era. *Genome Biol.* **3(2)**, 61 (2002).
5. Onyenwoke, R.U., Brill, J.A., Farahi, K., Wiegel, J. Sporulation genes in members of the low G+C Gram-type-positive phylogenetic branch (Firmicutes). *Arch Microbiol.* **182(2-3)**, 182-192 (2004).
6. Delmont, T.O. *et al.* Accessing the soil metagenome for studies of microbial diversity. *Appl Environ Microbiol.* **77(4)**, 1315-1324 (2011).
7. Ricca, E., Henriques, A.O., Cutting, S.M. *Bacterial spore formers: probiotics and emerging applications.* Horizon Bioscience. (2004).
8. Kuske, C.R. *et al.* Small-Scale DNA Sample Preparation Method for Field PCR Detection of Microbial Cells and Spores in Soil. *Appl Environ Microbiol.* **64(7)**, 2463-2472 (1998).
9. von Mering, C. *et al.* Quantitative phylogenetic assessment of microbial communities in diverse environments. *Science.* **315(8515)**, 1126-1130 (2007).

10. Wunderlin, T., Junier, T., Roussel-Delif, L., Jeanneret, N., & Junier, P. Stage 0 sporulation gene A as a molecular marker to study diversity of endospore-forming Firmicutes. *Environ Microbiol Rep.* **5(6)**, 911-924 (2013).
11. Siala, A., Hill, I.R., Gray, T.R.G. Populations of spore-forming bacteria in an acid forest soil, with special reference to *Bacillus subtilis. J General Microbiol.* **81(1)**, 183-190 (1974).
12. Brandes Ammann, A., Kolle, L., Brandl, H. Detection of bacterial endospores in soil by terbium fluorescence. *Int J Microbiol.* **2011**, 435281 (2011).
13. Fichtel, J., Koster, J., Rullkotter, J., Saas, H. High variations in endospore numbers within tidal flat sediments revealed by quantification of dipicolinic acid. *Geomicrobiol J.* **25(7-8)** (2008).
14. Cronin, U.P., Wilkinson, M.G. The potential of flow cytometry in the study of *Bacillus cereus. J Appl Microbiol.* **108(1)**, 1-16 (2010).
15. de Rezende, J.R. *et al.* Dispersal of thermophilic *Desulfotomaculum* endospores into Baltic Sea sediments over thousands of years. *ISME J.* **7(1)**, 72-84 (2013).
16. Hubert, C. *et al.* Thermophilic anaerobes in Arctic marine sediments induced to mineralize complex organic matter at high temperature. *Environ Microbiol.* **12(4)**, 1089-1104 (2010).
17. Garbeva, P., van Veen, J.A., van Elsas, J.D. Predominant *Bacillus* spp. in agricultural soil under different management regimes detected via PCR-DGGE. *Microb Ecol.* **45(3)**, 302-316 (2003).
18. Brill, J.A., Wiegel, J. Differentiation between spore-forming and asporogenic bacteria using a PCR and southern hybridization based method. *J Microbiol Methods.* **31(1-2)**, 29-36 (1997).
19. Rueckert, A., Ronimus, R.S., Morgan, H.W. Development of a real-time PCR assay targeting the sporulation gene, *spo0A.*, for the enumeration of thermophilic bacilli in milk powder. *Food Microbiol.* **23(3)**, 220-230 (2006).
20. Bueche, M. *et al.* Quantification of endospore-forming firmicutes by quantitative PCR with the functional gene *spo0A. Appl Environ Microbiol.* **79(17)**, 5302-5312 (2013).
21. Wunderlin, T., Junier, T., Roussel-Delif, L., Jeanneret, N., Junier, P. Endospore-enriched sequencing approach reveals unprecedented diversity of Firmicutes in sediments. *Environ Microbiol Rep.* **6(6)**, 631-639 (2014).
22. Nicholson, W.L., Law, J.F. Method for purification of bacterial endospores from soils: UV resistance of natural Sonoran desert soil populations of *Bacillus* spp. with reference to *B. subtilis* strain 168. *J Microbiol Methods.* **35(1)**, 13-21 (1999).

8.2 Comparison of the plant growth promotion performance of a consortium of Bacilli inoculated as endospores or as vegetative cells

Isha Hashmi, Christophe Paul, Andrej Al-Dourobi, Frederic Sandoz, Priscilla Deschamps, Thomas Junier, Pilar Junier, Saskia Bindschedler.

2019, FEMS Microbiology Ecology (Vol. 95, n°11, pp. 1-14), DOI: 10.1093/femsec/fiz147

Foreword

This study aimed to evaluate the effect of the inoculation of a bacterial consortium on the plant growth promotion. Both the comparative efficiency of single strain versus consortium, and the application of the inoculant under the form of vegetative cells or as endospores was tested. Many studies evaluated the effect of such inoculation on the plant growth, but relatively little is known about its effect on the native communities of microorganisms, bacteria and fungi included. Such studies are of main importance for the development of new biological plant growth promoting agents, without perturbating the natural environment and the indigenous communities, which could have undesirable effect on the soil diversity and fertility.

My contribution was the analysis of the sequencing data, and the analysis of bacterial and fungal communities. I contributed to the writing of the submitted manuscript, for the parts concerning the sequencing and community analysis, and also as a reviewer.

FEMS Microbiology Ecology, 95, 2019, fiz147

doi: 10.1093/femsec/fiz147
Advance Access Publication Date: 12 September 2019
Research Article

RESEARCH ARTICLE

Comparison of the plant growth-promotion performance of a consortium of Bacilli inoculated as endospores or as vegetative cells

Isha Hashmi[1], Christophe Paul[1], Andrej Al-Dourobi[1], Frederic Sandoz[1], Priscilla Deschamps[1], Thomas Junier[1,2], Pilar Junier[1] and Saskia Bindschedler[1,*]

[1]Laboratory of Microbiology, University of Neuchâtel, 2000 Neuchâtel, Switzerland and [2]Vital-IT Group, Swiss Institute of Bioinformatics, 1015 Lausanne, Switzerland

*Corresponding author: Laboratory of Microbiology, University of Neuchâtel, Rue Emile-Argand 11, Neuchâtel, Switzerland. Tel: +41 32 718 25 71; Fax: +41 32 718 30 01; E-mail: Saskia.bindschedler@unine.ch

One sentence summary: Plant growth promotion resulting from the use of beneficial bacteria was more effective when using a combination of different bacteria, rather than individual strains. This image was created with BioRender.

Editor: Paolina Garbeva

ABSTRACT

The effect of three plant growth-promoting *Bacillus* strains inoculated either alone or as a consortium was tested on oat (*Avena sativa*) growth. The bioinoculants were applied as vegetative cells or endospores at low cell densities on the seeds and their effect was tested in sterile *in vitro* conditions, pot experiments, and a field trial. The *in vitro* seed germination assay showed that both individual bacterial inocula and bacterial consortia had positive effects on seed germination. Greenhouse pot experiments with sterile and non-sterile soil showed that consortia increased the total dry biomass of oat plants as compared to single strain inoculation and uninoculated controls. However, the positive impact on plant growth was less prominent when the bioinoculated strains had to compete with native soil microbes. Finally, the field experiment demonstrated that the consortium of vegetative cells was more efficient in promoting oat growth than the endospore consortium and the uninoculated control. Moreover, both consortia successfully colonized the roots and the rhizosphere of oat plants, without modifying the overall structure of the autochthonous soil microbial communities.

Keywords: bacterial consortia; *Bacillus thuringiensis*; *Bacillus licheniformis*; *Avena sativa*; plant growth promotion; native microbial communities

INTRODUCTION

Global population growth is putting tremendous pressure on agriculture because of the decrease in arable land and the concomitant increase in the demand for a reliable food supply. Additionally, inappropriate agricultural techniques, such as intensive exploitation of soil, have considerably reduced soil fertility (Souza, Ambrosini and Passaglia 2015). In conventional agriculture, competitive food production is maintained through the continuous use of agrochemicals to enhance soil fertility, crop yield, and to control diseases. However, this has resulted in leaching or runoff of agrochemicals into groundwater, rivers and lakes, creating low oxygen zones where survival of aquatic life is at risk (Muhibbullah, Salma and Chowdhury 2005). Moreover, direct and indirect exposure to hazardous chemicals has

Received: 5 March 2019; **Accepted:** 10 September 2019

deleterious effects on the health of humans and the environment (Muhibbullah, Salma and Chowdhury 2005). Another drawback of agrochemical use is that uptake efficiency by plants decreases over time, requiring regular applications along with increased production cost for farmers. Moreover, for some nutrients, application does not result in higher bioavailability of nutrients. For instance, it has been reported that phosphorous fertilizers precipitate with metal cations and turn into an insoluble form that is no longer bioavailable to plants (Ahemad and Kibret 2014). Therefore, a change in these practices and innovative ways of conducting sustainable food production are a clear need for the future of agriculture.

Many studies have shown the potential of inoculation with plant growth-promoting (PGP) microorganisms to improve agricultural yield (Bhattacharyya and Jha 2012; Ahemad and Kibret 2014; Souza, Ambrosini and Passaglia 2015). More recently, bacterial consortia (mixtures of PGP bacterial strains) were shown to have higher performances as compared to the inoculation of individual species (Baez-Rogelio *et al.* 2017). For instance, a consortium of PGP bacteria induced the production of defense compounds (such as proline, ascorbate peroxidase or catalase) in response to the biotic stress caused by the phytopathogenic fungus *Fusarium oxysporum* (Akhtar *et al.* 2016). Bacterial consortia were also shown to promote plant drought tolerance (Wang *et al.* 2012). In beans, a consortium of *Rhizobium tropici* and two strains of *Paenibacillus polymyxa* increased growth and helped to alleviate the effect of abiotic drought stress as compared to single strain inoculation (Figueiredo *et al.* 2008).

Bioinoculation of PGP microorganisms also presents many challenges. One of the critical issues that needs to be addressed for application in the field is the survival of bacteria acclimated under laboratory conditions to the harsh conditions in soils. In order to persist in soils, inoculated microorganisms have to compete with autochthonous microbial communities (Souza, Ambrosini and Passaglia 2015). Bioinoculants are also vulnerable to the scarce nutrient availability in soil, as compared to rich nutrient conditions usually provided by growth media, and ultimately declines in the abundance of inoculated bacteria within the soil are frequently reported (Trabelsi and Mhamdi 2013; Souza, Ambrosini and Passaglia 2015). Moreover, the delivery of these microorganisms in an active form is an additional challenge. Application of carrier materials for the protection of bioinoculants (for instance, karnolite, peat or charcoal) has proven to be environmentally unfriendly, as well as costly, making this approach inapplicable as a general practice in agriculture (Arora, Tiwar and Singh 2014). These concerns are the incentive to identify effective bacterial inoculants to be applied in bioinoculation technology.

Among the currently used PGP bacteria, *Bacillus* is one of the best-studied examples. There are many species of *Bacillus* that are well known as plant growth-promoters (Kumar, Prakash and Johri 2011). One of the most important characteristics of this genus is that they form endospores, which are a resistant structure that fosters survival. This property can be harnessed in bioinoculation technologies, as it results in long shelf-life of the product before application. Moreover, *Bacillus* spp., followed by *Enterobacter* spp. and *Pseudomonas* spp., are the most abundant bacteria with PGP traits in the rhizosphere of plants grown in arid conditions because of their high tolerance to extreme environmental conditions (El-Sayed *et al.* 2014). *Bacillus* spp. are also used in phytoremediation technologies, increasing biomass production and multi-metal accumulation in plants (Chibuike and Obiora 2014). *Bacillus* spp. can help plants by not only stimulating their growth through increasing nutrient acquisition (e.g. phosphate solubilization, atmospheric nitrogen fixation, phytohormone and siderophore production (Bhattacharyya and Jha 2012)) but also acting as biocontrol agents against various pests (O'Callaghan 2016; Widnyana and Javandira 2016). *Bacillus* spp. can also produce a wide range of antiviral, antibacterial and antifungal compounds, which may be important in their interactions with plants and other soil microorganisms. In addition, it has been reported that many species of *Bacillus* induce systemic resistance in plants against a broad spectrum of phytopathogens (Nui *et al.* 2011)

In this study, a consortium consisting of three *Bacillus* strains was used to promote the growth of oat (*Avena sativa*) plants. The strains were initially tested for physiological traits linked to plant growth-promoting activities. Next, their ability to promote plant growth was assessed in experiments at four levels of complexity (i.e. *in vitro* seed germination under sterile conditions, greenhouse pot experiments with sterile substrate and non-sterile soil, and finally in a field trial). We hypothesized that based on the complementarity of the plant growth-promoting traits of the strains, the consortium will provide a more robust effect on plant growth, as compared to single strain inoculation. This should represent a real advantage when going from controlled laboratory to field conditions, where the inoculated PGP bacteria will compete with the autochthonous microbial community. Additionally, we also compared two forms of bacterial inocula: endospores and vegetative cells, to assess whether dormancy alters the functionality of the consortium. Finally, we investigated the effect of bioinoculation on native bacterial and fungal communities in the field assay. We hypothesized that a consortium applied directly onto the seeds at low cell densities (ca. 10^3 cells per oat seed) should not affect the indigenous soil microbial communities.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The bacterial strains *Bacillus thuringiensis* 1312 (BT1) and *B. thuringiensis* 1310 (BT2) were isolated from soils of the Atacama Desert, Chile, during a sampling campaign carried out by the laboratory of microbiology, University of Neuchâtel in 2011. *Bacillus licheniformis* (BL) was isolated from soil at Agroscope Liebefeld, Bern, Switzerland. The strains were cryopreserved in 60% glycerol and when required for the experiment, the strains were precultured in nutrient agar (NA, Carl Roth, Karlsruhe, Germany) media.

Physiological characterization of bacterial strains

Dinitrogen fixation assay

A loop of an overnight bacterial culture on NA was streaked onto a nitrogen-free medium (Döbereiner 1980), which was then incubated at 30°C for 48–72 h. Bacteria were cultured over three generations on the nitrogen-free medium and the capacity to fix N_2 was assessed by the growth of bacterial strains in the third-generation plates.

Casein solubilization (proteolysis)

Casein solubilization was assessed based on a modified protocol from Frazier and Rupp (1931). This assay was used as a proxy for the ability of the selected *Bacillus* strains to solubilize organic nitrogen from proteins. The medium was composed of 5% skimmed milk powder (Migros, Zürich, Switzerland), 1.2% malt extract powder (SIOS, Wald, Switzerland) and 1.5% agar

(Merck, Darmstadt, Germany). A loop of an overnight grown bacterial culture on NA was streaked onto the casein-solubilizing medium. The plates were incubated for 48–72 h at 30°C. A transparent halo observed around a bacterial colony indicated that the strain was able to solubilize casein.

Siderophore production assay

To assess the ability of the three bacterial strains to chelate iron, a siderophore production test was performed as described in Schwyn and Neilands (1987). Briefly, the initial medium has a blue coloration resulting from the complexation of ferric iron with chrome azurol S (CAS, Thermo Fischer Scientific, Waltham MA, USA) and hexadecyltrimethylammonium bromide (HDTMA, Merck, Darmstadt, Germany). When a strong iron chelator (such as a siderophore) removes iron from the dye complex, the color of the medium turns to yellow. A loop of an overnight bacterial culture on NA was inoculated onto the medium. The plates were incubated at 30°C and a change of color was assessed after 48–72 h.

Auxin-like phytohormone production assay

To check for the production of auxin-like compounds, a modified version of the protocol proposed by Bric, Bostock and Silverstone (1991) was used. In this assay, the Angle medium (Angle, Mcgrath and Chaney 1991) was supplemented with 5 mM Tryptophane (Merck, Darmstadt, Germany), as a precursor for auxin biosynthesis. The test was performed in 96-well microplates with 270 μL of medium per well. A loop of an overnight bacterial culture from NA medium was inoculated in the three microplate wells for each corresponding bacterial strain. The microplate was then incubated at 25°C for 72 h in the dark. After 72 h, one drop of Salkowski's reagent was added to each well and placed in dark for 45 min. A pink to red coloration indicates the production of auxin-like compounds.

Bacterial compatibility

Compatibility of the strains developing as a consortium was tested by growing them together on a favorable medium (NA, Carl Roth, Karlsruhe, Germany). Each bacterial strain was refreshed overnight in 25 mL nutreint medium and 2 μL inocula containing 10^6 bacterial cells of each strain was placed on a Petri dish containing NA medium. Each strain was inoculated 1 cm apart from other strains in the Petri dish and the plates were incubated at 30°C for 72 h. The experiment was performed in four replicates. Inhibition was assessed visually using overall culture images and close-ups of overlapping areas of the expanding colonies. An additional approach using CRISPR-Cas9 technology to tag the strains with fluorescent proteins was tested in order to visualize and count the cells individually, but this was unsuccessful until now.

Preparation of bacterial inoculants

Seed bioinoculation with the three selected bacterial strains was performed using either vegetative cells or endospores, and both either as individual cells or as a consortium.

Vegetative cells treatment

Pure cultures of the three selected bacterial strains on nutrient agar (NA, Carl Roth, Karlsruhe, Germany) were used to individually inoculate 50 mL of nutrient broth (NB, Carl Roth, Germany) medium which was further incubated overnight at 30°C and 150 rpm (HT incubator, Infors AG, Bottmingen, Switzerland). An overnight bacterial culture of each strain was transferred into a sterile 50 mL tube and centrifuged (2–16PK, Sigma, Osterode am Harz, Germany) at 2150 g for 3 min. The resulting pellet was washed five times with sterile physiological water. After this, the optical density of each bacterial suspension was measured at 550 nm (Genesys 10S UV-VIS spectrophotometer, Thermo Fisher Scientific, Waltham MA, USA) and adjusted to reach a final concentration of 10^6 colony forming units (CFU) mL^{-1}. The resulting suspension of vegetative cells was used for experiments with plants (*in vitro* assay of seed germination, greenhouse pot experiments and field experiment). This resulted in four different treatments for experiments with vegetative cells: VBT1 = *B. thuringiensis* 1312, VBT2 = *B. thuringiensis* 1310, VBL = *B. licheniformis* and vegetative cells consortium = VM.

Endospore treatment

A synthetic medium was used to induce sporulation of the bacterial strains (sporulation medium; Donnellan, Nags and Levinson 1964). Each bacterial strain was refreshed in 50 mL NB. Overnight bacterial cultures were centrifuged (2–16PK, Sigma, Osterode am Harz, Germany) at 7673 g for 3 min. The resulting pellet was washed five times with physiological water. At the end, the pellet of each bacterial strain was suspended in 100 mL of sporulation medium and incubated for 3 weeks at 30°C and 200 rpm. After 3 weeks, endospore formation was verified by phase contrast microscopy. Vegetative cells observed in the suspension were killed by a heat shock at 70°C for 15 min for *B. thuringiensis* 1312 (BT1) and *B. thuringiensis* 1310 (BT2) and at 80°C for 10 min for *B. licheniformis* (BL). Afterward, endospores of each bacterial strain were collected by centrifugation at 7673 g for 10 min. The resulting pellet was suspended in 2 mL of sterile distilled water. The quantities of spores were estimated using the Neubauer Chamber cell counting method and dilutions were made and adjusted to reach a final concentration of 10^5 spores mL^{-1}. The endospore suspensions were then used in the *in vitro* assay of seed germination, greenhouse pot experiments, as well as in the field experiment. This resulted in four different treatments for experiments with endospores: SBT1 = *B. thuringiensis* 1312, SBT2 = *B. thuringiensis* 1310, SBL = *B. licheniformis* and endospore consortium = SM.

Seed inoculation with bacterial inoculants

Oat seeds (*A. sativa*) were soaked in 5% sodium hypochlorite (NaClO) solution for 2 min and then thoroughly rinsed five times with sterile distilled water. Sterilized seeds were incubated for 30 min and placed on a shaker at 150 rpm in the eight respective bacterial treatment suspensions (i.e. VBT1, VBT2, VBL, VM, SBT1, SBT2, SBL and SM) under sterile conditions in order to allow for the cells or spores to adhere to the seeds. Control treatment consisted in surface sterilized seeds treated in the same way, but using sterile distilled water instead of bacterial suspensions.

Bacterial adhesion onto seeds

To assess bacterial adhesion onto seeds (each of the eight different treatments and the control), three seeds were randomly sampled in order to count bacterial cells or endospores adhering at their surface by flow cytometry. Seeds of each individual treatment were placed in sterile Eppendorf tubes containing 2 mL of sodium hexametaphosphate $(NaPO_3)_6$ and shaken vigorously with a vortex mixer for few seconds. For the vegetative cell treatment, 10 μL of SYBR Green was added to fluorescently

label the cells. This treatment was not applied for endospore treatments because SYBR Green is unable to stain dormant cells (Zheng, Xiong and Wu 2017).

In addition to flow cytometry, scanning electron microscopy (SEM) was used to assess adhesion of vegetative cells or endospores onto the seeds. Uninoculated control seeds and seeds treated with both consortia (vegetative cells or endospores) were prefixed in 2.5% glutaraldehyde (Merck, Darmstadt, Germany) for 1 h followed by washing twice with sterile distilled water. Secondary fixation was carried out by using 4% osmium tetroxide (OsO_4, Merck, Darmstadt, Germany) for 1 h, followed by two washing steps with sterile distilled water. Then, stepwise dehydration in graded alcohol was carried out by incubating samples for 15 min at each of the following ethanol concentrations (50%, 75%, 90% and 100%; Merck, Darmstadt, Germany). After this, the samples were dipped in tetramethysilane ($C_4H_{12}Si$; TMS, Merck, Darmstadt, Germany) for 20 min with a second step of 1 h until complete TMS evaporation. The samples were mounted onto SEM stubs covered with graphite and coated with gold with a sputter coater (Bal-Tec sputter coater SCD 005). The seeds were then examined using high-vacuum SEM (Philips ESEM XL30 FEG, Philips, Amsterdam, Netherlands) at an acceleration of 10 KeV and a working distance of ~8.6 mm.

In vitro seed germination assay

In order to determine the effect of the different bacterial treatments on oat seed germination, an *in vitro* assay was performed. Five seeds inoculated with each of the eight bacterial treatments were grown aseptically on sterile filter paper. Filter papers were kept moist with sterile distilled water and incubated at 22°C in the dark. Uninoculated seeds were used as control. Experiments were replicated five times such that 25 seeds were assessed for each treatment. After 10 days, the number of germinating seeds was counted, and percentage of seed germination was calculated based on the number of seeds that germinated divided by the total number of seeds placed in Petri dishes.

Greenhouse pot experiments

Pot experiments were conducted in a greenhouse located at the University of Neuchâtel, Switzerland, to study the effect of each of the eight bacterial treatments (i.e. VBT1, VBT2, VBL, VM, SBT1, SBT2, SBL and SM) on growth of oat plants in soil. Two different experiments were carried out, either with a sterile substrate or a non-sterile soil, in order to compare plant growth-promoting activities of the different type of inoculation (single strain inocula versus consortium, as well as vegetative cells versus endospores). This approach was used to observe how the inoculants perform in a simple (sterile substrate) and a more complex (non-sterile soil) environment. The sterile substrate was composed of 25% peat, 25% Seramis® Pflanz-granulat (clay granules; Seramis, Mogendorf, Germany) and 50% sand (mixed carbonate and silicate; Coop, Basel, Switzerland). A thin layer of this substrate was sterilized by five successive autoclave cycles. Sterile substrate was then added in sterile pots (122 g in 130 mL pots). For the non-sterile treatment, soil was collected from a grassland nearby the University of Neuchâtel, Switzerland. The soil corresponded to a calcaric Cambisol (WRB 2015). Pots corresponding to nine treatments (eight bacterial treatments and an uninoculated control treatment) were arranged randomly in the greenhouse. One seed was planted in each pot. For the sterile substrate, 7 replicates per treatment were performed, while for non-sterile soil 10 replicates were performed. A larger number of replicates in the second case were considered given the potential for larger variability in the results when using soil as substrate. Pots were irrigated regularly with 0.5× concentrated Murashige and Skoog (MS, Duchefa Biochemie, Haarlem, Netherlands) solution for the sterile artificial substrate and tap water for the non-sterile soil. The numbers of seedlings that germinated and eventually grew into plants that were used for the experiment are indicated in Table S1 (Supporting Information). Plants from each individual treatment were harvested after 45 days. Plants (shoots, roots, flowers and pods) were dried at 60°C for 72 h and their total dry weight determined using a fine-scale balance.

Field experiment

To study the effect of both consortia, composed of either vegetative cells or endospores, on oat plants growth, a field experiment was conducted at a designated research field of the University of Neuchâtel, Switzerland. This was carried out in collaboration with the GRAMU association (Groupe d'Aménagement de l'Unine) involved in various projects related to sustainable agriculture and permaculture. Based on the results of the greenhouse pot experiments, we selected only three treatments for the field experiment: an uninoculated control, the vegetative cells consortium (VM) and the endospores consortium (SM). The selection of a limited number of treatments was made to allow for enough replication of the experiments in the field. The total area of the field was 5 m × 1.35 m, which was subdivided into nine plots to get three replicated plots of each treatment (Figure S1, Supporting Information). The position of the plots for each treatment was randomized. Each plot consisted of a length of 40 cm with a consecutive gap of 18 cm in which no treatment was applied. In each subdivided plot, 23 seeds were initially sown. The final numbers of seedlings that germinated and eventually grew into a plant and that were used for the experiment are indicated in Table S1 (Supporting Information). Plots were irrigated regularly, and weeds were manually removed. Plants (consisting of shoots, roots and seeds) were harvested after 85 days and dried at 60°C for 72 h. The total dry weight of the plants was measured using a fine-scale balance and the number of seeds per plant were counted. Physicochemical analyses (pH, water content and water holding capacity, concentrations in bioavailable and total phosphorus, nitrates, ammonium, organic carbon as well as calcium carbonate) were carried out on the soil of the field experiment to characterize its fertility. These measurements were done before and after the experiment for each of the three treatments (control, VM and SM; Table S2, Supporting Information).

DNA extraction

In order to study the effect of bioinoculation on native bacterial and fungal soil communities in the field experiment, different samples (bulk soil, rhizospheric soil and roots) were compared. Bulk soil consisted of soil that was distant from the roots influence. This fraction was analyzed before sowing and after harvesting. Rhizospheric soil consisted of the soil that was adhering to the roots upon sampling (Luster *et al.* 2009), and was obtained by detaching soil from the roots by vigorously shaking them. Finally, to obtain the root sample, the roots were washed with

sodium hexametaphosphate ($(NaPO_3)_6$; Merck, Darmstadt, Germany) to remove any remaining soil particles. With this treatment, we assumed that microbes strongly attached to the root surface, as well as root endophytes were present (Reinhold-Hurek and Hurek 2011). DNA extraction was performed in triplicates for each treatment. DNA was obtained from 3 g of soil (bulk and rhizospheric soil) and 0.5 g of root samples. Fungal DNA was obtained by following the protocol of the FastDNA®SPIN kit for soil according to the manufacturer's instructions (MP Biomedicals, Waltham MA, USA; direct DNA extraction). Bacterial DNA was obtained using an indirect DNA extraction method previously developed to enhance the extraction of resistant structures such as endospores (Wunderlin *et al.* 2013). This method consists of an initial pre-extraction from the environmental samples, resuspending the soil twice in 10 mL of 1% $(NaPO_3)_6$ for the initial pre-extraction step (indirect method), followed by a modified DNA extraction protocol with the FastDNA®SPIN kit for soil (MP Biomedicals, Waltham MA, USA), with three successive bead-beating steps in the initial part of the protocol. DNA concentrations were measured with a Qubit Fluorometer using the dsDNA HS Assay Kit (Invitrogen, Carlsbad CA, USA). The concentration of all samples was adjusted to 2 ng μL^{-1} by diluting with double distilled sterile water.

Microbial community analyses

Amplicon sequencing was performed on an Illumina MiSeq platform, using the services of Fasteris SA (Geneva, Switzerland). For the bacterial community analysis, the regions V3-V4 of the 16S rRNA gene were targeted using universal primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3') (Herlemann *et al.* 2011). For the fungal community analysis, the 18S rRNA gene was targeted using the primer pair AMV4.5NF (5'-AAGCTCGTAGTTGAATTTCG-3') and AMDGR (5'-CCCAACTATCCCTATTAATCAT-3'), which have been specifically designed for the analysis of arbuscular mycorrhizal fungal communities (AMF; Glomeromycota) (Sato *et al.* 2005). In total 36 samples were sent for sequencing of both markers. However, for the bacterial V3-V4 region, sequences were obtained only for 31 samples. The five missing samples consisted in one replicate of the control and two replicates of VM-treated post-harvest bulk soils, as well as two replicates of the root fraction controls.

The Illumina reads were processed with Mothur (Schloss *et al.* 2009) following the MiSeq standard procedure (Kozich *et al.* 2013), and using the SILVA NR v123 (16S rRNA) and SILVA NR v132 (18S rRNA) reference databases (Quast *et al.* 2013) for the alignment of amplicons and the taxonomic assignment of representative OTUs (operational taxonomic unit). Chimeras and singletons were removed prior to OTU clustering. For the 16S rRNA sequencing, average neighbor clustering of the 535 919 retained sequences (79 430 unique sequences) at 97% identity led to the identification of 6475 OTUs. For the 18S rRNA sequencing, clustering the 7 978 155 retained sequences (54 754 unique sequences) at 97% identity using the opticlust algorithm (Westcott and Schloss 2017) led to the identification of 11 466 OTUs. In addition, as a proxy for the detection of the inoculated strains at the end of the experiment, the bacterial sequences were used to query for near to exact matches to the 16S rRNA gene sequences of the inoculated strains. Bacterial sequences were queried using the strains 16S rRNA gene fragment matching the sequenced region. Those sequences were extracted from the draft genomes of the three strains. The Smith–Waterman algorithm (Smith and Waterman 1981) was used to check for near identical matches (identity of 99% or above). The number of sequences matching the queries under this stringent cut-off was used to calculate the relative abundance of the inoculated strains in the different samples.

Although the primers used for analyzing the fungal community were specifically designed for the analysis of AMF (Sato *et al.* 2005), sequences assigned to other divisions were also found to be highly represented, mostly in the Basidiomycota (Figure S2, Supporting Information). This contrasts with a previous study evaluating the potential of using these primers for targeting the AMF community in root samples, which showed a high specificity of these primers (Van Geel *et al.* 2014). The differences in the soil compartments analyzed (bulk soil, rhizosphere soil and roots in the present study) and the use of different sequencing technologies with different sequencing depths (Miseq VS 454 pyrosequencing) might partly explain these results. Sequences were submitted to GenBank under the Bioproject accession numbers PRJNA472865 for bacterial and fungal communities.

Statistical analysis

All statistical analyses were computed using R (version 3.4.3) (R Core Team 2014). Plant data from the greenhouse pots and field experiments were analyzed for statistical significance ($P < 0.05$) using one-way analysis of variance (ANOVA) followed by a Tukey's *post hoc* test. Comparisons of *in vitro* seed germination were analyzed with binomial test followed by Tukey's *post hoc* test. Bacterial and fungal community analyses were performed using phyloseq and vegan packages (McMurdie and Holmes 2013; Oksanen *et al.* 2017). Bacterial and fungal communities were analyzed by principal coordinates analysis (PCoA), based on Bray–Curtis dissimilarity and Hellinger transformation of the OTU table. OTUs accounting for less than four reads in the whole dataset were removed prior to the analysis. Comparisons between groups (soil compartments and treatments) were performed by permutational multivariate analysis of variance (PERMANOVA) using the adonis function from the vegan package. Analysis was based on the same dissimilarity matrix as described above, with 5000 permutations. Additional pairwise comparisons were performed using the function pairwise.adonis from the package pairwise adonis (Martinez Arbizu 2019), with 5000 permutations and Holm correction. Finally, the effect of treatment was analyzed with and without controlling for the soil compartment (use of the 'strata' parameter in the adonis function).

RESULTS

Screening of plant growth-promoting activities of the three bacterial strains

The screening for PGP traits of the three bacterial strains showed that all of the strains possessed all of the tested traits. All three strains were able to grow on nitrogen-free medium, showing their ability to fix atmospheric nitrogen. They were also able to utilize organic nitrogen, as demonstrated by their ability to solubilize casein. Moreover, all of them produced siderophores and auxin-like phytohormone compounds. Furthermore, the three stains were able to grow as a consortium as it was observed that the individual growth of each strain was not affected when grown as a co-culture under laboratory conditions (Figure S3, Supporting Information).

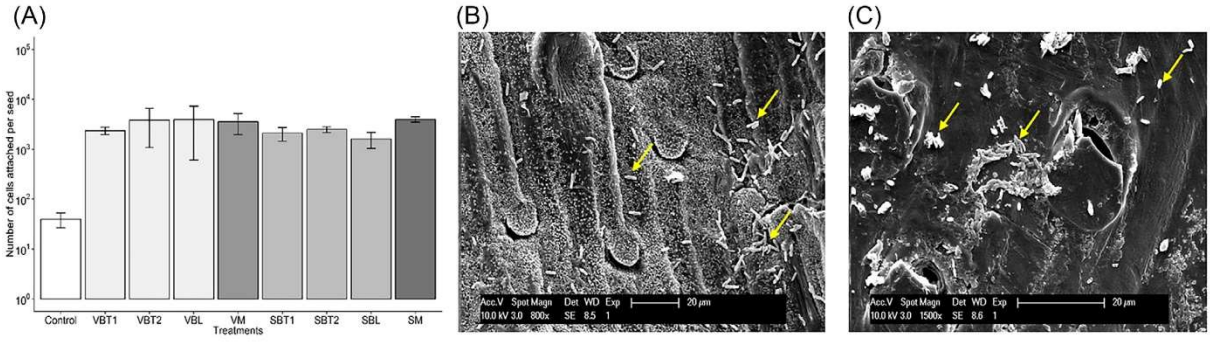


Figure 1. Assessment of the number of cells or spores attaching to *A. sativa* seeds after bioinoculation. **(A)** Number of vegetative cells or endospores attached per seed measured by flow cytometry (Control = uninoculated; V = vegetative cells; S = endospores; BT1 = *B. thuringiensis* 1312; BT2 = *B. thuringiensis* 1310; BL = *B. licheniformis*; Consortium = M). SEM images of seeds bioinoculated with either **(B)** the consortium of vegetative cells or **(C)** the consortium of endospores. Arrows show bacterial cells or spores attached onto the seeds.

Assessment of bacterial inoculants adhesion onto seeds

The attachment of bacterial cells onto the seeds was quantitatively measured by flow cytometry. The number of cells attached per seed was in the range of 10^3 cells/seed (Fig. 1A). A slightly higher number of cells was observed on seeds treated with vegetative cells as compared to endospores, with the exception of the endospore consortium. The number of cells on seeds treated with the consortium of either vegetative cells (VM) or endospores (SM) was also assessed qualitatively by SEM observation (Fig. 1B and C). The number of bacterial cells attached to the seeds was higher for the treatment with vegetative cells compared to the seeds treated with endospores (Table S3, Supporting Information). No bacterial cells were observed on the untreated control seeds.

In vitro seed germination assay

The *in vitro* seed germination assay showed that all three bacterial strains enhanced seed germination. Inoculation solely with vegetative cells of *B. thuringiensis* 1312 (VBT1) had the highest effect on seed germination (92%), which was significantly (adj $P < 0.05$) different from the control (52% of seed germination) and other treatments with vegetative cells (Fig. 2A; Table S4, Supporting Information). The vegetative cell consortium (VM) also induced higher germination than the untreated control (72% seed germination), although this difference was not statistically significant. In seeds treated with endospores, a trend of increased seed germination was observed in all treatments. However, only the treatment with *B. thuringiensis* 1310 (SBT2; 92%) and the consortium (96%) were significantly different from the control (Fig. 2B; Table S4, Supporting Information). Overall, the percentage of germinating seeds increased with inoculation using either individual strains or consortia as compared to the uninoculated control treatment.

Greenhouse pot experiments

The effect of individual strains on the total dry weight of oat plants was not statistically different from the untreated control treatment regardless of the mode of inoculation (vegetative cells or endospores) (Fig. 3). This result was consistent in experiments using both a sterile substrate (Fig. 3A) and a non-sterile soil (Fig. 3B). In contrast, inoculation with a consortium of the three selected *Bacillus* strains had a positive effect on plant growth, and this was the case for both the vegetative cells and endospore consortia. However, this effect was less prominent in the experiments with non-sterile soil as compared to the sterile substrate (Fig. 3). Inoculation with consortia showed a significant increase (adj $P < 0.05$; Table S5, Supporting Information) in total dry weight of oat plants compared to uninoculated control plants. No significant difference was observed when comparing the type of consortium (vegetative cells or endospores; Fig. 3; Table S5, Supporting Information).

Field experiment

Given the results obtained in the greenhouse experiments, only the consortia were evaluated in the field experiment. The plants inoculated with the vegetative cell consortium (VM) showed a significant increase in total dry weight compared to the uninoculated control (Fig. 4A; Table S6, Supporting Information). The plants treated with the VM consortium also produced significantly more seeds compared to the uninoculated control (Fig. 4B). Inoculation with the vegetative cell consortium (VM) did not show a significantly greater beneficial effect of plant growth promotion compared to the endospores consortium (SM).

Microbial community analyses

The effect of bioinoculation with both consortia on the autochthonous bacterial and fungal communities in different soil compartments (pre-sowing and post-harvest bulk soil, rhizospheric soil and roots) was evaluated. PCoA computed from the bacterial communities (Fig. 5) exhibited four significant axes according to the Kaiser–Guttman criterion, while the broken stick model retained only the first axis, indicating that axis 2 of the PCoA must be interpreted with caution. Axis 1 of the PCoA explained 34.4% of the variance and clearly separated two groups of samples: roots from bulk (pre-sowing and post-harvest) and rhizospheric soil (Fig. 5). Samples from the pre-sown bulk soil grouped together, indicating that the community composition was homogenous at the beginning of the experiment. Although there was some variability between samples from the rhizospheric and the post-harvest bulk soils (illustrated by their arrangement along the axis 2), there was no clear separation between samples from these soil compartments based on the PCoA. Comparing the three treatments (i.e. untreated

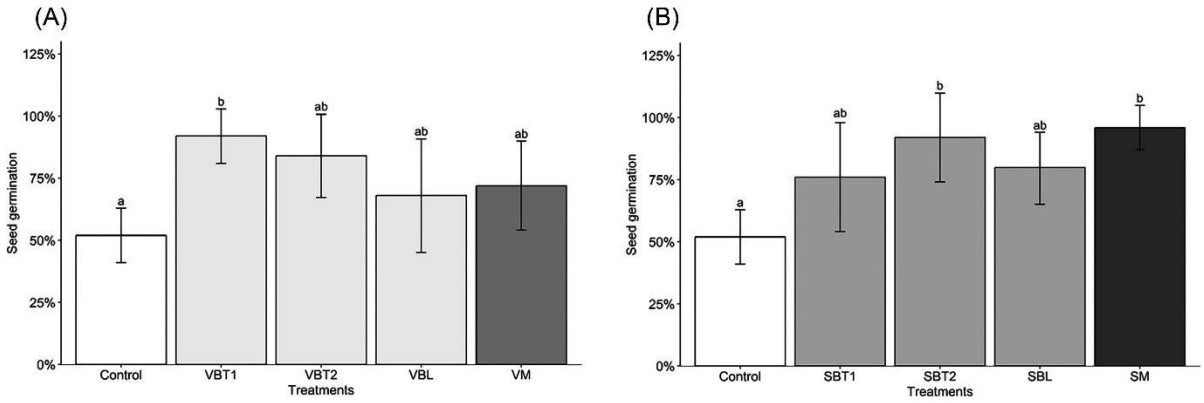


Figure 2. Percentage of *A. sativa* seed germination comparing the effect of seeds treated with individual inocula and consortia of both (A) vegetative cells and (B) endospores. Different lowercase letters denote statistically significant differences among different treatments of bacterial inoculation at the 5% level according to Tukey's *post hoc* test ($n = 5$). Control = uninoculated; V = vegetative cells; S = endospores; BT1 = *B. thuringiensis* 1312; BT2 = *B. thuringiensis* 1310; BL = *B. licheniformis*; consortium = M.

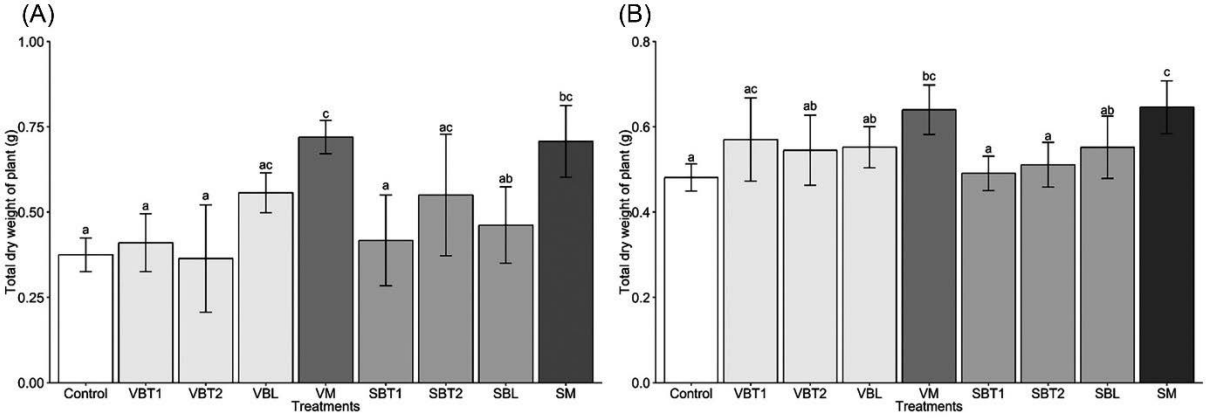


Figure 3. Effects of individual inocula and both consortia (vegetative cells and endospores) on total dry weight of *A. sativa* grown in pots using (A) a sterilized substrate ($n = 3$ to 7; Table S1, Supporting Information) and (B) a non-sterilized soil ($n = 8$ to 10; Table S1, Supporting Information). The bar represents the average dry weight of the number of replicates per treatment (Table S1, Supporting Information) and error bars indicate standard deviation to this average value. Different lowercase letters denote statistically significant differences among different treatments of bacterial inoculation at the 5% level according to Tukey's *post hoc* test. Control = uninoculated; V = vegetative cells; S = endospores; BT1 = *B. thuringiensis* 1312; BT2 = *B. thuringiensis* 1310; BL = *B. licheniformis*; consortium = M.

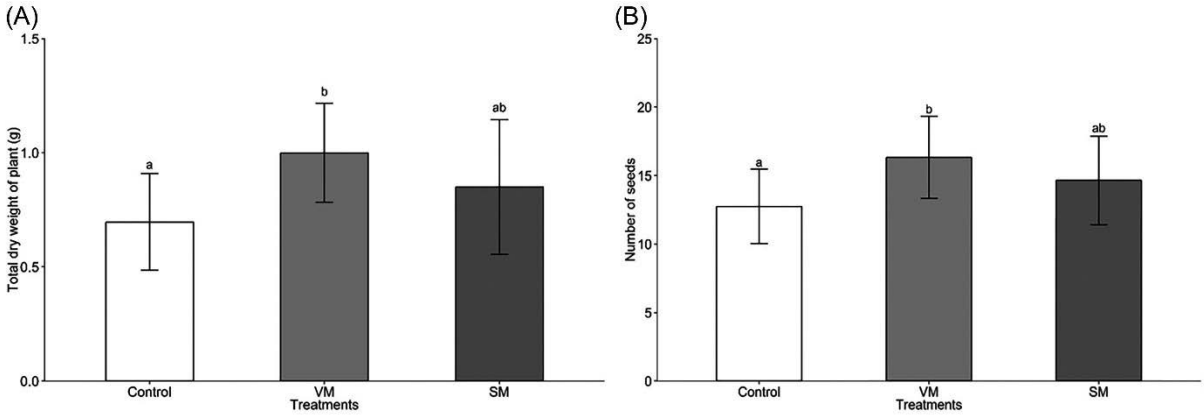


Figure 4. Plant growth-promoting effects of both consortia (vegetative cells and endospores) on *A. sativa* plants grown in the field. (A) Total dry weight of oat plants and (B) total number of seeds produced per oat plant. The bars represents average dry weight and number of seeds of 28 plants (the number of plants in individual plots appears in Table S1, Supporting Information) in each respective treatment. Error bars represent standard deviation to the average value. Different lowercase letters denote statistically significant differences among different treatments of bacterial inoculation at the 5% level according to Tukey's *post hoc* test ($n = 28$). Control = uninoculated; VM = consortium of vegetative cells; SM = consortium of endospores.

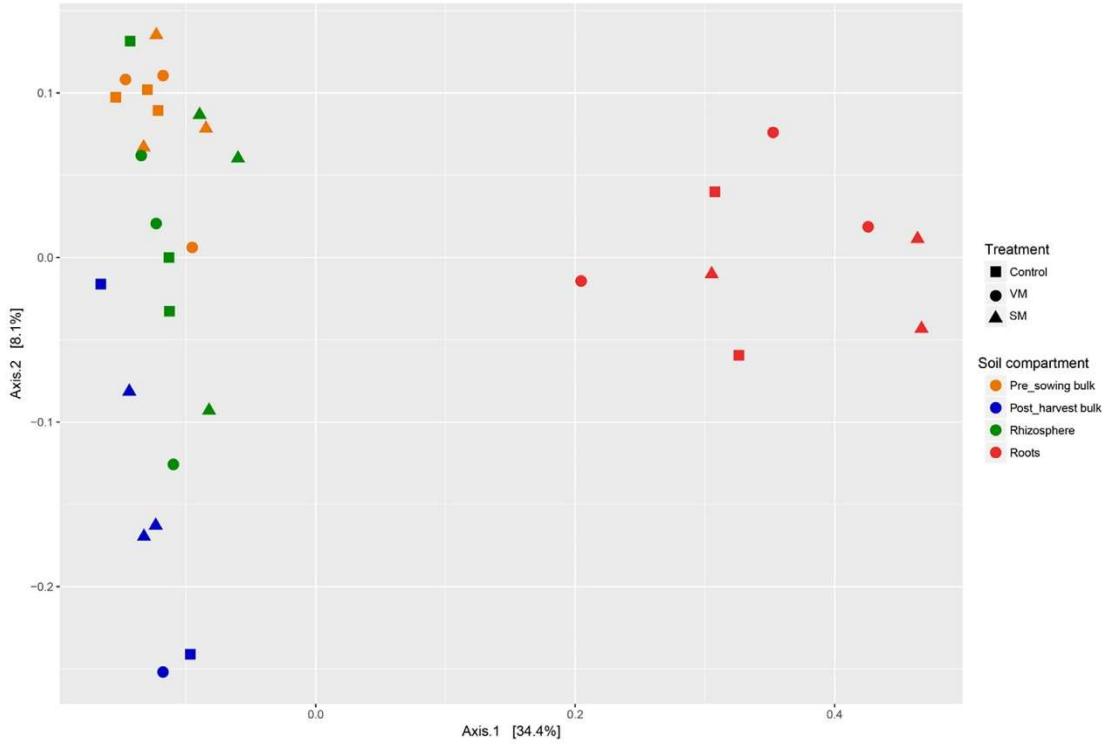


Figure 5. PCoA based on Bray–Curtis dissimilarity of bacterial community composition in bulk soils (pre-sowing and post-harvest), rhizospheric soil and roots. Percentage of the variation between the samples explained by each axis is indicated on the respective axis. The shape of the symbol represents the different treatments (Control = uninoculated; VM = consortium of vegetative cells; SM = consortium of endospores). Colors represent the different soil compartments. For the post-harvest bulk soil libraries prepared for the control and VM treatment, only two and one replicate yielded sequence information, respectively. The same was true for root samples in the control treatment, in which only two replicates were included in the analysis.

control, inoculation with either the vegetative cell consortium (VM) or endospore consortium (SM)), no prominent difference was observed regardless of the soil compartment considered. A PERMANOVA analysis confirmed that the community was significantly different across the soil compartments, each pairwise comparison between groups being significant (Table S7, Supporting Information). On the contrary, comparison based on the treatment did not show any significant difference (Table S7, Supporting Information).

The relative abundance of OTUs assigned to *Bacillus* spp. was also analyzed (Fig. 6). OTUs affiliated to *Bacillus* spp. represented only a minor fraction of the community (3–6%). The highest relative abundance was observed in the case of the rhizosphere of VM-treated plants (Fig. 6). A BLAST search with the OTUs corresponding to *Bacillus* showed that all of them were between 96% and 100% identical to *B. thuringiensis* or *B. licheniformis* (Table S8, Supporting Information). Moreover, a refined search was performed in order to identify identical sequences corresponding to the 16S rRNA gene of the inoculated strains. We found a 5–8-fold potential increase of the inoculated strains in rhizosphere (VM treatment), post-harvest and root (SM treatment) compartments, relative to the pre-sown conditions (Figure S4, Supporting Information).

Analysis of the fungal communities yielded a similar trend to the one observed for the bacterial communities. Only the first PCoA axis was significant based on the broken stick model, compared to 11 axes determined using the Kaiser–Guttman criterion. Axis 1 of the PCoA explained 22.7% of the variance and separated the same two groups of community components: root-associated fungi versus soil-associated fungi (including rhizospheric soil; Fig. 7). Similar to the bacterial communities, fungal communities in the pre-sown bulk soil appeared homogenous. Samples from the rhizospheric and post-harvest bulk soils did not show a clear separation according to the soil compartment. Although PCoA tended to group samples belonging to the same soil compartment, some samples fell out of these groups. Moreover, no clear distinction was made based on the different treatments (i.e. untreated control, inoculation with the vegetative cell consortium (VM) or inoculation with the endospore consortium (SM)). Results of the PERMANOVA on the fungal communities are similar to those from the bacterial communities, and confirmed the observations made from the PCoA: communities are significantly different across the soil compartments, while treatment had no significant effect on the fungal community composition (Table S7, Supporting Information).

DISCUSSION

In this study, a consortium of three *Bacillus* strains showed plant growth-promoting activities *in vitro* and they were also effective in promoting the growth of oat plants both in pots (sterile substrate and non-sterile soils) and in the field. There are several studies investigating the effect of bacterial inoculation on plant growth, either directly in the field or by comparing their efficiency under controlled conditions (García *et al.* 2004; Mishra *et al.* 2009; Ali *et al.* 2011; Sharma, Ramesh and Johri 2013;

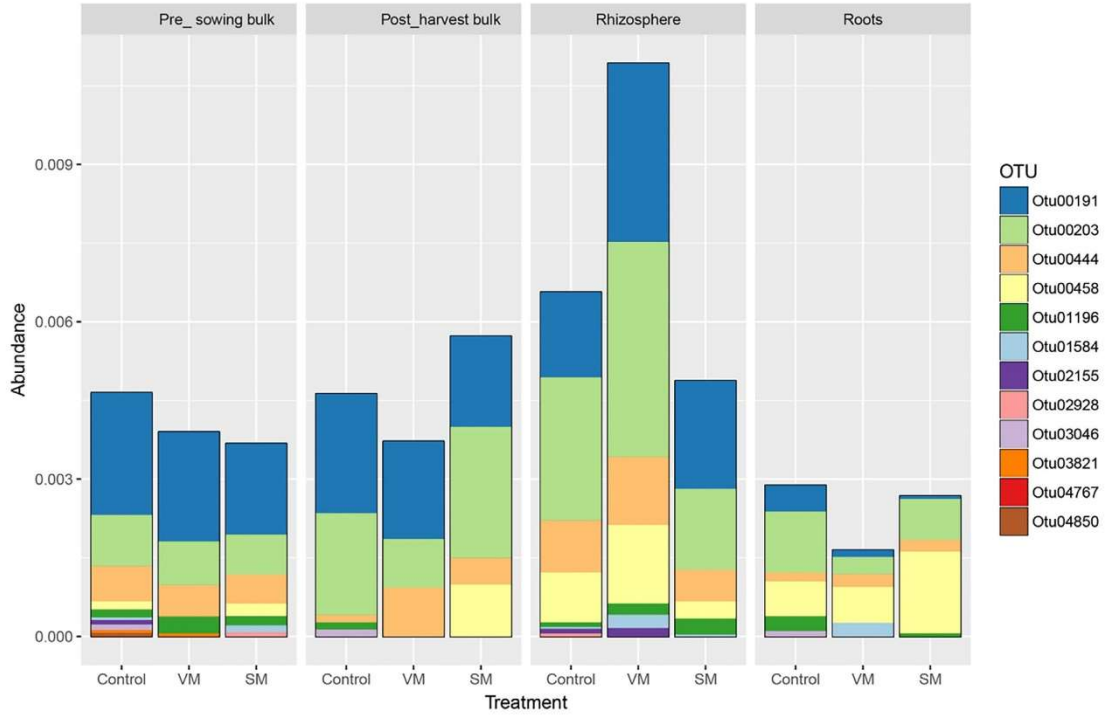


Figure 6. Relative abundance of the 12 OTUs assigned to *Bacillus* spp. in pre-sowing bulk soil (bulk soil before the experiment), post-harvest bulk soil (bulk soil after the experiment), rhizospheric soil and roots. The different colors represent the different OTUs.

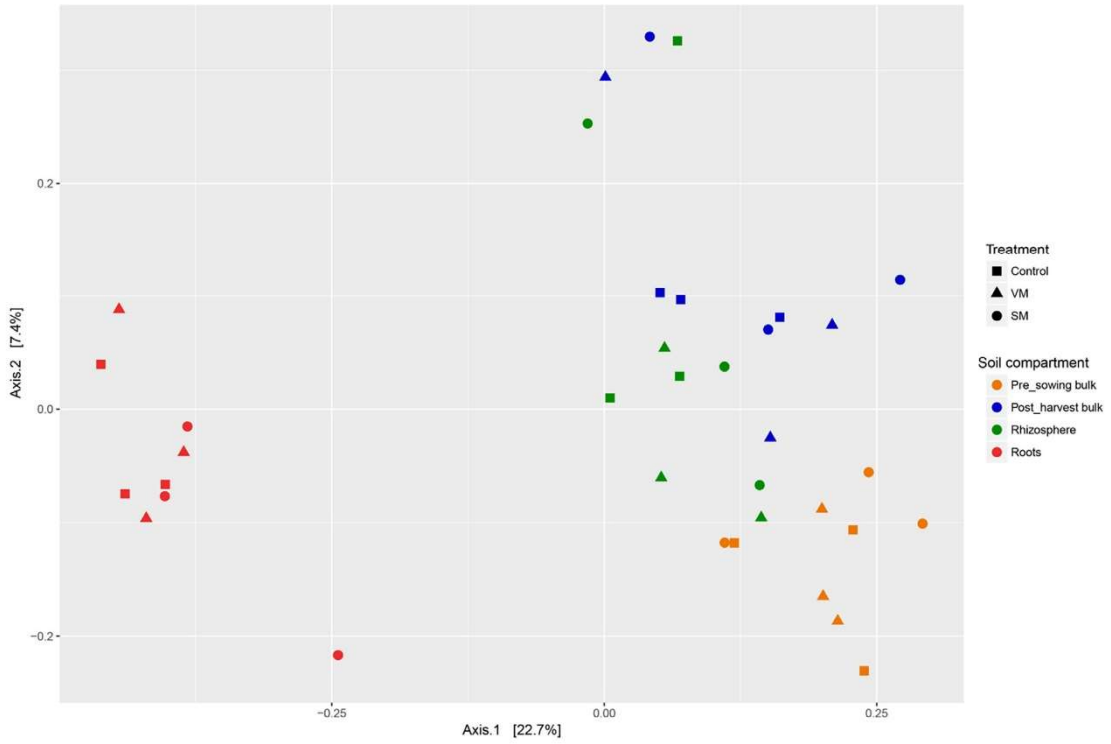


Figure 7. PCoA based on Bray–Curtis dissimilarity of fungal community composition in bulk soils (pre-sowing and post-harvest), rhizospheric soil and roots. Percentage of the variation between the samples explained by each axis is indicated on the respective axis. The shape of the symbol represents the different treatments (Control = uninoculated; VM = consortium of vegetative cells; SM = consortium of endospores) and the colors represent the different soil compartments.

Masciarelli, Llanes and Luna 2014; Kuan *et al.* 2016; Samaniego-Gámez *et al.* 2016; Yasmin *et al.* 2016; Hernández-Montiel *et al.* 2017). However, there are fewer systematic studies that span from the selection of bacterial inoculant to testing their effect on seed germination and also testing their ability to promote plant growth under different conditions, from sterile substrate and non-sterile soil in controlled condition to field trials. Likewise, evaluating the effect of microbial inoculants on native bacterial and fungal communities is seldomly done. Therefore, to the best of our knowledge, this study presents the first thorough investigation that combines both approaches.

This study started with the *in vitro* screening of plant growth-promoting traits in 15 mesophilic *Bacillus* strains of different origins from the University of Neuchâtel microbiology laboratory's bacterial collection (data not presented). Among these bacterial strains, three strains, BT1, BT2 and BL, showed positive results for a maximal number of traits and were thus selected for further *in planta* experiments, irrespective of the fact that two of them originated from Chile (Atacama Desert) and were identified as belonging to the same species. The key element that was considered for the selection was functional redundancy (multiple strains providing the same plant growth-promoting trait), rather than their phylogenetic affiliation. It may be assumed that two strains of a same species (as well as closely related species) are functionally similar. However, it is well known that defining a species in bacteria is difficult, in particular in the genomic era (Konstantinidis, Ramette and Tiedje 2006), and that strains of a similar species may have completely different metabolic capabilities (Wielbo *et al.* 2010). Furthermore, functional redundancy is defined as the 'ability of one microbial taxon to carry out a process at the same rate as another under the same environmental conditions' (Allison and Martiny 2008). Therefore, we hypothesized that using closely related species within a consortium is a trade-off that has the advantage of having some genetic variation, while ensuring functional redundancy in plant promoting traits. While functional redundancy is a concept that is widely recognized in microbial ecology, to the best of our knowledge, it is rarely used with reference to the inoculation of plant growth-promoting microbes in the form of a consortium.

Inoculation of oat seeds with single strains or a consortium of three bacteria increased germination rate as compared to uninoculated seeds. Therefore, direct seed coating seems to have beneficial effects on seed germination. This could be due to the ability of the selected strains to produce auxin-like hormones. There are many reports showing that *Bacillus* spp. are able to enhance plant growth by producing different plant growth hormones such as gibberellins, indole acetic acid (IAA) and cytokinins (Arkhipova *et al.* 2005; Radhakrishnan and Lee 2016). It has also been reported that some *Bacillus* spp. were able to produce more than one growth hormone, with a beneficial effect on the overall physiology of plants (Kumar, Prakash and Johri 2011). Moreover, we choose seed inoculation over foliar spray because the former introduces the required inoculants directly into the soil at the vicinity of the emerging roots. When the roots develop, beneficial bacteria are already present to stimulate plant growth. It has been reported that inoculation of seeds reduces seed-borne diseases caused by soil phytopathogens (O'Callaghan 2016). Furthermore, according to the findings of Ciccillo *et al.* (2002), the application method and density of the bacterial inoculants play a vital role on plant growth promotion. For instance, incorporation in soil of different concentrations (10^6, 10^7 and 10^8 CFU g^{-1} soil) of the plant growth-promoting strain *Burkholderia ambifaria* MCI 7 had a negative effect on plant growth, with the strongest reduction observed at 10^8 CFU g^{-1} soil. In the same study, same plant growth-promoting strain had a significant positive effect on the growth of maize plants when applied directly onto seeds at similar concentrations (10^6, 10^7 and 10^8 CFU per seed). However, bacterial inoculum concentration was not a significant predictor of plant biomass (Ciccillo *et al.* 2002). In our study, we found that oat seeds carried $\sim 10^3$ cells of inoculated bacteria. Nonetheless, even at such low cell density, a positive effect toward oat growth was observed. This result is in accordance with a previous study where an inoculum of *Bacillus amyloliquefaciens* BNM122B was used for seed coating of soybean for protection against the plant pathogen *Rhizoctonia solani* (Correa *et al.* 2009). In that experiment, the initial concentration of the bacterial suspension was 10^8 CFU mL^{-1} and the final concentration of bacterial cells attached per seed was 10^4 CFU, showing that even at low cell densities the desired outcome can be obtained.

One of the most significant results obtained in the present study is that inoculation with a consortium of bacteria had a more robust effect on oat biomass than inoculation with individual bacterial strains in the pot experiments. Similar observations were also obtained in other studies using combined bioinoculants. For instance, a consortium of two *Bacillus* strains and *Enterobacter cloacae* increased tomato seedling growth and helped in disease suppression (Abdeljalil and Renault 2016). Moreover, it has been reported that inoculation with a consortium of *R. tropici* and two different strains of *P. polymyxa* elicits plant growth in presence of an abiotic stress (drought), in contrast to inoculation with *R. tropici* alone (Figueiredo *et al.* 2008). Finally, (Jetiyanon 2007) showed that a consortium of *Bacillus* spp. alleviated biotic stress caused by phytopathogens by producing defense-related enzymes.

In the field experiment, the consortia of either vegetative cells or endospores were compared, without including the comparison of individual strains. This was partly due to logistic limitations such as space and time to replicate plots at an agronomic scale. The choice of comparing both life forms rather than single inocula *versus* consortium was rationalized by the fact that in the greenhouse pot experiments in which nine different treatments were compared, consortia appeared to have more robust and consistent effects than single strains, whatever the cell type used (vegetative cells or endospores). Therefore, in the field experiment, the comparison of the two life forms of *Bacillus* cells and their effectiveness in plant growth promotion was investigated. This is of particular interest since *Bacillus*-based commercial products, for instance Kodiak, Rhizovital®42, FZB24® *Bacillus subtilis*, Serenade® and Quantum-400, contain in most cases spores, which are supposed to enhance the shelf life of commercial products (Radhakrishnan, Hashem and Abd Allah 2017).

In our study, we found that the bacterial inoculum applied in the form of vegetative cells had a more prominent effect in terms of total dry weight and grain yield of oat plants compared to the inoculum containing endospores, even though plants inoculated with endospores still grew larger than the untreated control plants. In a study comparing inoculation with vegetative cells and endospores of *Bacillus subtilis* EA-CB0575 (at concentration of 1×10^7 and 1×10^8 CFU mL^{-1}, respectively), both the vegetative cells and endospores enhanced total dry weight of banana plants as compared to untreated controls (Posada *et al.* 2016). This suggests that both cellular forms can be equally used for bioinoculation technology. However, germination of spores in the soil is crucial for the strain to work effectively. In our study, the inoculation with endospores was less

effective than the inoculation with vegetative cells and we hypothesized that this was the result of a low germination of the inoculated strains. Petras and Casida (1985) demonstrated that the germination of *B. thuringiensis* spores in the soil can be poor. They also noticed that despite the fact that spores had the ability to survive in the soil, germination was poor, and spores only germinated when plated on a nutritive medium in the laboratory. Likewise, Crane, Frodyma and Bergstrom (2014) observed that spores of *B. amyloliquefaciens* were unable to inhibit the growth of *Fusarium graminearum* (responsible for the fungal disease *Fusarium* head blight) on wheat. To overcome this problem, the authors suggested using nutrients to induce spore germination (a mixture composed of D-glucose, D-fructose and potassium chloride, along with amino acids, either L-asparagine or L-alanine). Finally, Omer (2010) compared a bioformulation of *Bacillus megatherium* and free endospore suspensions, finding that in field applications, an inorganic carrier increased the shelf life of the product and the bioformulation of *B. megatherium* with a phosphate stabilizer enhanced the viability and thus the efficacy of spores, as well as the growth promotion of bean plants, compared to the free endospore suspensions. Therefore, these studies demonstrate that spore germination is not always guaranteed in the field and that investigating this aspect is of interest to allow developing bioformulations with enhanced efficacy in the field.

Besides assessing the beneficial effect of bioinoculation on plant growth, another aspect often neglected in studies dealing with the use of bioinoculants is the impact of this practice on the indigenous soil microflora (Orhan *et al.* 2006; Noumavo *et al.* 2013; Kuan *et al.* 2016; Widnyana and Javandira 2016; Korir *et al.* 2017; Moustaine *et al.* 2017). Investigating whether the applied consortium has an impact on the structure of the native microbial community is crucial for allowing further usage of the inoculum in replacement of agrochemicals in sustainable agricultural ecosystems. It is important to consider that bioinoculants could potentially have a major impact on the long-term functional capabilities of the resident microbial communities by changing their overall structure (Trabelsi and Mhamdi 2013). Several studies have reported that bacterial inoculants alter the bacterial community composition after inoculation (Schwieger and Tebbe 2000; García de Salamone *et al.* 2012; Trabelsi *et al.* 2012). Although a previous study has shown that the plant growth-promoting rhizobacteria *Bacillus* sp. SUT1 and *Pseudomonas* sp. SUT19 did not alter the native fungal communities of Chinese broccoli (Piromyou *et al.* 2013), more recently, it was reported that inoculation of three *Bacillus* strains (*B. cereus*, *B. subtilis* and *B. amyloliquefaciens*) changed the endosphere bacterial communities of broccoli (Gadhave *et al.* 2018). In some cases, drastic shifts were observed, for instance, two biocontrol agents *Pseudomonas corrugate* IDV1 and *Pseudomonas putida* RA2, changed the composition of the autochthonous bacterial community from a Gram-positive- to a Gram-negative-dominated population in the rhizosphere of maize (Kozdrój, Trevors and van Elsas 2004). Such effects should not be ignored. One of the main factors explaining drastic changes in the resident bacterial community could be the high cell densities (up to 10^9 CFU mL^{-1}) used in bioinoculation experiments (Kozdrój, Trevors and van Elsas 2004). In our study, we found that using a low bacterial cell density (10^3 cells per seeds) for the direct application of the inoculant onto seeds effectively increased the growth of oat plants, with no significant effect on the structure of the native bacterial and fungal communities (Figs 5 and 7). The *Bacillus* spp. fraction remained a minor fraction of the bacterial community even after the experiment and some of these community members were likely the bioinoculated strains as suggested by the outcome of the BLAST search (Fig. 6; Figure S4, Supporting Information). This again demonstrates that the bioinoculated strains were able to colonize the rhizosphere of oat plants, but not to outcompete native bacteria.

Importantly, the bacterial and fungal communities associated to the roots were found to be highly different from the communities of the other soil compartments (bulk and rhizospheric soils), showing that oat roots had a unique root-associated community. This is in accordance with previous studies investigating the differences in the microbiome of roots and other soil compartments. This was true for the bacterial microbiomes of the roots and the surrounding soil of maize (Niu *et al.* 2017). Similar observations regarding fungi were reported by Urbina *et al.* (2018) in a study comparing roots, rhizospheric and bulk soil fungal communities. Finally, both fungal and bacterial communities associated with *Populus* differed between roots and rhizospheric soil (Gottel *et al.* 2011).

CONCLUSION

In summary, a consortium of three *Bacillus* strains with plant growth-promoting activities positively affected oat seed germination and plant growth. This effect was less prominent in non-sterile soil as compared to a sterile substrate, highlighting the importance of competition with the native microbial communities and demonstrating that inoculated consortia can provide more robust plant growth promotion than single strain inocula. In sterile conditions (*in vitro* seed germination experiment an pot experiment with sterile substrate), consortia of both vegetative cells and endospores performed similarly. Conversely, in non-sterile conditions (pot experiment with non-sterile soil and field trial), vegetative cells showed slightly higher performances in promoting plant growth. Moreover, we also show that direct seed inoculation with bacterial consortia at low cell density did not lead to an alteration of the indigenous bacterial and fungal communities. Indeed, it appeared that the bioinoculated strains actually established in the rhizosphere of oat plants, but without a major effect on native microbial communities. This is of particular importance since a change in the indigenous communities induced by the inoculation could lead to unpredictable and undesirable effects on plant and ecosystem health. Therefore, we conclude that a consortium of three different *Bacillus* strains could be used as a low-cost and effective alternative for improving growth of oat plants. Finally, even though vegetative cells performed slightly better than endospores, both type of cells may be used to promote plant growth. This study may be helpful in the field of sustainable agriculture and could lead to more effective bacterial inoculants for promoting plant growth and health. Indeed, our results help expand the knowledge on how microbial consortia interact with plants and soil native microbial communities. This promotes a deeper and more comprehensive understanding of the factors required for the development and the formulation of next-generation bacterial inoculants to be used for sustainable agricultural ecosystems.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

FUNDING

This work was supported by: the Swiss Government Excellence Scholarships for Foreign Scholars (number 2014.0865 to

IH), the University of Neuchâtel Swiss National Science Foundation overheads (grant number 11.1 to SB), the 'Fondation Pierre Mercier pour la Science' (to SB), and the U.S. Department of Energy, Office of Science, Biological and Environmental Research Division, under award number LANLF59T (to PJ).

ACKNOWLEDGEMENTS

We would like to thank Geoffrey House for proofing the English language of the revised version of the manuscript.

Conflicts of interest. None declared.

REFERENCES

Abdeljalil NOB, Renault D. Evaluation of the effectiveness of tomato-associated rhizobacteria applied singly or as three-strain consortium for biosuppression of sclerotinia stem rot in tomato. *J Microbial Biochem Technol* 2016;**8**:312–320.

Ahemad M, Kibret M. Mechanisms and applications of plant growth promoting rhizobacteria: current perspective. *Science* 2014;**26**:1–20.

Akhtar N, Naveed M, Muhammad Zafar I *et al.* Effect of consortium of plant growth promoting and compost inhabiting bacteria on physicochemical changes and defense response of maize in fungus infested soil. *J Agr Sci* 2016;**53**:59–68.

Ali SZ, Sandhya V, Grover M *et al.* Effect of inoculation with a thermotolerant plant growth promoting Pseudomonas putida strain AKMP7 on growth of wheat (Triticum spp.) under heat stress. *J Plant Interact* 2011;**6**:239–46.

Allison SD, Martiny JBH. Resistance, resilience, and redundancy in microbial communities. *Proc Natl Acad Sci* 2008;**105**:11512–9.

Angle JS, Mcgrath SP, Chaney RL. New culture medium containing ionic concentrations of nutrients similar to concentrations found in the soil solution. *Appl Environ Microb* 1991;**57**:3674–76.

Arkhipova TN, Veselov SU, Melentiev AI *et al.* Ability of bacterium Bacillus subtilis to produce cytokinins and to influence the growth and endogenous hormone content of lettuce plants. *Plant Soil* 2005;**272**:201–09.

Arora NK, Tiwar S, Singh R. Comparative study of different carriers inoculated with nodule forming and free living plant growth promoting bacteria suitable for sustainable agriculture. *J Plant Pathol Microb* 2014;**05**:1000229.

Baez-Rogelio A, Morales-García YE, Quintero-Hernández V *et al.* Next generation of microbial inoculants for agriculture and bioremediation. *Microb Biotechnol* 2017;**10**:19–21.

Bhattacharyya PN, Jha DK. Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. *World J Microbiol Biotechnol* 2012;**28**:1327–50.

Bric JM, Bostock RM, Silverstone SE. Rapid in situ assay for indoleacetic acid production by bacteria immobilized on a nitrocellulose membrane. *Appl Environ Microb* 1991;**57**:535–38.

Chibuike GU, Obiora SC. Heavy metal polluted soils: effect on plants and bioremediation methods. *Appli Environ Soil Sci* 2014;**2014**:1–12.

Ciccillo F, Fiore A, Bevivino A *et al.* Effects of two different application methods of Burkholderia ambifaria MCI 7 on plant growth and rhizospheric bacterial diversity. *Environ Microbiol* 2002;**4**:238–45.

Correa OS, Montecchia MS, Berti MF *et al.* Bacillus amyloliquefaciens BNM122, a potential microbial biocontrol agent applied on soybean seeds, causes a minor impact on rhizosphere and soil microbial communities. *Appl Soil Ecol* 2009;**41**:185–94.

Crane JM, Frodyma ME, Bergstrom GC. Nutrient-induced spore germination of a Bacillus amyloliquefaciens biocontrol agent on wheat spikes. *J Appl Microbiol* 2014;**116**:1572–83.

Donnellan JE, Nags EH, Levinson HS. Chemically defined, synthetic media for sporulation and for germination and growth of bacillus subtilis. *J Bacteriol* 1964;**87**:332–36.

Döbereiner J. Forage grasses and grain crops. *Methods for Evaluating Biological Nitrogen Fixation*. New York: John Wiley & Sons Ltd, 1980, 535–55.

El-Sayed WS, Akhkha A, El-Naggar MY *et al.* In vitro antagonistic activity, plant growth promoting traits and phylogenetic affiliation of rhizobacteria associated with wild plants grown in arid soil. *Front Microbiol* 2014;**5**:651.

Figueiredo MVB, Burity HA, Martínez CR *et al.* Alleviation of drought stress in the common bean (Phaseolus vulgaris L.) by co-inoculation with Paenibacillus polymyxa and Rhizobium tropici. *Appl Soil Ecol* 2008;**40**:182–88.

Frazier WC, Rupp P. Studies on the proteolytic bacteria of milk: v. action of proteolytic bacteria on milk serum. *J Bacteriol* 1931;**21**:263–71.

Gadhave KR, Devlin PF, Ebertz A *et al.* Soil inoculation with Bacillus spp. modifies root endophytic bacterial diversity, evenness, and community composition in a context-specific manner. *Microb Ecol* 2018;**76**:741–50.

García de Salamone IE, Funes JM, Di Salvo LP *et al.* Inoculation of paddy rice with Azospirillum brasilense and Pseudomonas fluorescens: impact of plant genotypes on rhizosphere microbial communities and field crop production. *Appl Soil Ecol* 2012;**61**:196–204.

García JAL, Probanza A, Ramos B *et al.* Effect of inoculation of Bacillus licheniformison tomato and pepper. *Agronomie* 2004;**24**:169–76.

Gottel NR, Castro HF, Kerley M *et al.* Distinct microbial communities within the endosphere and rhizosphere of *populus deltoides* roots across contrasting soil types. *Appl Environ Microb* 2011;**77**:5934.

Herlemann DP, Labrenz M, Jürgens K *et al.* Transitions in bacterial communities along the 2000 km salinity gradient of the Baltic Sea. *ISME J* 2011;**5**: 1571–9.

Hernández-Montiel LG, Chiquito Contreras CJ, Murillo Amador B *et al.* Efficiency of two inoculation methods of Pseudomonas putida on growth and yield of tomato plants. *J Soil Sci Plant Nut* 2017;**17**:1003–12.

Jetiyanon K. Defensive-related enzyme response in plants treated with a mixture of Bacillus strains (IN937a and IN937b) against different pathogens. *Biol Control* 2007;**42**:178–85.

Konstantinidis KT, Ramette A, Tiedje JM. The bacterial species definition in the genomic era. *Philos Trans R Soc Lond B Biol Sci* 2006;**361**:1929–40.

Korir H, Mungai NW, Thuita M *et al.* Co-inoculation effect of rhizobia and plant growth promoting rhizobacteria on common bean growth in a low phosphorus soil. *Front Plant Sci* 2017;**8**:141.

Kozdrój J, Trevors JT, van Elsas JD. Influence of introduced potential biocontrol agents on maize seedling growth and bacterial community structure in the rhizosphere. *Soil Biol Biochem* 2004;**36**:1775–84.

Kozich JJ, Westcott SL, Baxter NT *et al.* Development of a dual-index sequencing strategy and curation pipeline for

analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Appl Environ Microbiol* 2013;**79**:5112–20.

Kuan KB, Othman R, Abdul Rahim K *et al.* Plant growth-promoting rhizobacteria inoculation to enhance vegetative growth, nitrogen fixation and nitrogen remobilisation of maize under greenhouse conditions. *PLoS One* 2016;**11**:e0152478.

Kumar A, Prakash A, Johri BN. Bacillus as PGPR in crop ecosystem. *Bacteria in Agrobiology: Crop Ecosystems*. Berlin, Heidelberg: Springer, 2011, 37–59.

Luster J, Göttlein A, Nowack B *et al.* Sampling, defining, characterising and modeling the rhizosphere—the soil science tool box. *Plant Soil* 2009;**321**:457–82.

Martinez Arbizu P. *pairwiseAdonis: Pairwise Multilevel Comparison Using Adonis*. R Package Version 0.3, 2019.

Masciarelli O, Llanes A, Luna V. A new PGPR co-inoculated with Bradyrhizobium japonicum enhances soybean nodulation. *Microbiol Res* 2014;**169**:609–15.

McMurdie PJ, Holmes S. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One* 2013;**8**:e61217.

Mishra PK, Mishra S, Selvakumar G *et al.* Enhanced soybean (Glycine max L.) plant growth and nodulation by Bradyrhizobium japonicum-SB1 in presence of Bacillus thuringiensis-KR1. *Acta Agr Scand B-S P* 2009;**59**:189–96.

Moustaine M, Elkahkahi R, Benbouazza A *et al.* Effect of plant growth promoting rhizobacterial (PGPR) inoculation on growth in tomato (Solanum Lycopersicum L.) and characterization for direct PGP abilities in Morocco. *IJEAB* 2017;**2**:590–96.

Muhibbullah M, Salma M, Chowdhury A. Use of agrochemical fertilizers and their impact on soil, water and human health in the Khamargao Village of Mymensingh District, Bangladesh. *J Agron* 2005;**4**:109–15.

Niu B, Paulson JN, Zheng X *et al.* Simplified and representative bacterial community of maize roots. *Proc Natl Acad Sci USA* 2017;**114**:E2450–E59.

Noumavo PA, Kochoni E, Didagbé YO *et al.* Effect of different plant growth promoting rhizobacteria on maize seed germination and seedling development. *AJPS* 2013;**04**:1013–21.

Nui DD, Liu HX, Jiang CH *et al.* The plant growth–promoting Rhizobacterium Bacillus cereus AR156 induces systemic resistance in arabidopsis thaliana by simultaneously activating salicylate- and jasmonate/ethylene-dependent signaling pathways. *Mol Plant Microbe Interact* 2011;**24**:533–42.

O'Callaghan M. Microbial inoculation of seed for improved crop performance: issues and opportunities. *Appl Microbiol Biotechnol* 2016;**100**:5729–46.

Oksanen J, Blanchet FG, Friendly M *et al.* vegan: Community Ecology Package. Ordination methods, diversity analysis and other functions for community and vegetation ecologists. Version 2.4-4. 2017. URL: https://CRAN.R-project.org/package=vegan.

Omer AM. Bioformulations of Bacillus Spores for using as Biofertilizer. *Life Sci J* 2010;**7**:124–31.

Orhan E, Esitken A, Ercisli S *et al.* Effects of plant growth promoting rhizobacteria (PGPR) on yield, growth and nutrient contents in organically growing raspberry. *Sci Hortic* 2006;**111**:38–43.

Petras SF, Casida LE. Survival of Bacillus thuringiensis Spores in Soil. *Appl Environ Microb* 1985;**50**:1496–501.

Piromyou P, Noisangiam R, Uchiyama H *et al.* Indigenous microbial community structure in rhizosphere of chinese kale as affected by plant growth-promoting rhizobacteria inoculation. *Pedosphere* 2013;**23**:577–92.

Posada LF, Ramírez M, Ochoa-Gómez N *et al.* Bioprospecting of aerobic endospore-forming bacteria with biotechnological potential for growth promotion of banana plants. *Sci Hortic* 2016;**212**:81–90.

Quast C, Pruesse E, Yilmaz P *et al.* The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res* 2013;**41**:D590–6.

Radhakrishnan R, Hashem A, Abd Allah EF. Bacillus: A Biological Tool for Crop Improvement through Bio-Molecular Changes in Adverse Environments. *Front Physiol* 2017;**8**:667.

Radhakrishnan R, Lee IJ. Gibberellins producing Bacillus methylotrophicus KE2 supports plant growth and enhances nutritional metabolites and food values of lettuce. *Plant Physiol Biochem* 2016;**109**:181–89.

R Core Team. *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing, 2014.

Reinhold-Hurek B, Hurek T. Living inside plants: bacterial endophytes. *Curr Opin Plant Biol* 2011;**14**:435–43.

Samaniego-Gámez BY, Garruña R, Tun-Suárez JM *et al.* Bacillus spp. inoculation improves photosystem II efficiency and enhances photosynthesis in pepper plants. *Chil J Agr Res* 2016;**76**:409–16.

Sato K, Suyama Y, Saito M *et al.* A new primer for discrimination of arbuscular mycorrhizal fungi with polymerase chain reaction-denature gradient gel electrophoresis. *Grassland Sci* 2005;**51**:179–81.

Schloss PD, Westcott SL, Ryabin T *et al.* Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol* 2009;**75**:7537–41.

Schwieger F, Tebbe CC. American society for microbiology. *Appl Environ Microb* 2000;**66**:3556–65.

Schwyn B, Neilands B. Universal chemical assay for the detection and determination of siderophores. *Anal Biochem* 1987;**160**:47–56.

Sharma S, Ramesh A, Johri B. Isolation and characterization of plant growth-promoting Bacillus amyloliquefaciens strain sks_bnj_1 and its influence on rhizosphere soil properties and nutrition of soybean (Glycine max L. Merrill). *J Virol Microbiol* 2013;**2013**:1–19.

Smith TF, Waterman MS. Identification of common molecular subsequences. *J Mol Biol* 1981;**147**:195–7.

Souza Rd, Ambrosini A, Passaglia LM. Plant growth-promoting bacteria as inoculants in agricultural soils. *Genet Mol Biol* 2015;**38**:401–19.

Trabelsi D, Ben Ammar H, Mengoni A *et al.* Appraisal of the crop-rotation effect of rhizobial inoculation on potato cropping systems in relation to soil bacterial communities. *Soil Biol Biochem* 2012;**54**:1–6.

Trabelsi D, Mhamdi R. Microbial inoculants and their impact on soil microbial communities: a review. *BioMed Res Int* 2013;**2013**:863240.

Urbina H, Breed MF, Zhao W *et al.* Specificity in Arabidopsis thaliana recruitment of root fungal communities from soil and rhizosphere. *Fungal Biol* 2018;**122**:231–40.

Van Geel M, Busschaert P, Honnay O *et al.* Evaluation of six primer pairs targeting the nuclear rRNA operon for characterization of arbuscular mycorrhizal fungal (AMF) communities using 454 pyrosequencing. *J Microbiol. Methods* 2014;**106**:93–100.

Wang CJ, Yang W, Wang C *et al.* Induction of drought tolerance in cucumber plants by a consortium of three plant growth-promoting rhizobacterium strains. *PLoS One* 2012;**7**:e52565.

Westcott SL, Schloss PD. OptiClust, an Improved Method for Assigning Amplicon-Based Sequence Data to Operational Taxonomic Units. *mSphere* 2017; **2**:e00073–17.

Widnyana IK, Javandira C. Activities Pseudomonas spp. and Bacillus sp. to stimulate germination and seedling growth of tomato plants. *Agric Agric Sci Proc* 2016; **9**:419–23.

Wielbo J, Kuske J, Marek-Kozaczuk M *et al.* The competition between Rhizobium leguminosarum bv. viciae strains progresses until late stages of symbiosis. *Plant Soil* 2010;**337**:125–35.

WRB. *World Reference Base for Soil Resources 2014, Update 2015. International Soil Classification System for Naming Soils and Creating Legends for Soil Maps*. World Soil Resources Reports No. 106. Rome: FAO, 2015.10.1109/MCSE.2015.88

Wunderlin T, Junier T, Roussel-Delif L *et al.* Stage 0 sporulation gene A as a molecular marker to study diversity of endospore-forming Firmicutes. *Environ Microbiol Rep* 2013;**5**:911–24.

Yasmin S, Zaka A, Imran A *et al.* Plant growth promotion and suppression of bacterial leaf blight in rice by inoculated bacteria. *PLoS One* 2016;**11**:e0160688.10.1371/journal.pone.0160688

Zheng XL, Xiong ZQ, Wu JQ. The use of a simple flow cytometry method for rapid detection of spores in probiotic Bacillus licheniformis-containing tablets. *Food Sci Biotechnol* 2017;**26**:167–71.

8.3 Le microbiote du sédiment: une approche novatrice pour tracer la consommation passée d'antibiotiques

Pilar Junier, Christophe Paul, Thomas Junier

2018, Pipette (Nr 6, pp. 16-17)

Foreword

This short publication describes the application of the method based on the spore isolation method, for the study of antibiotic resistance genes in the environment.

My contribution to this paper was the production and analysis of the data demonstrating the possible use of spore-like structures for tracking ARGs in the environment. I also helped with the writing of the manuscript, however acting mainly as a reviewer.

Pilar Junier[1], Christophe Paul[1], Thomas Junier[1,2]

Le microbiote du sédiment: une approche novatrice pour tracer la consommation passée d'antibiotiques

La découverte des antibiotiques comme moyen de lutte contre les infections bactériennes fait partie des grands succès médicaux du XXe siècle. La pénicilline a été d'une telle importance pour l'humanité qu'elle a conduit à l'octroi de deux Prix Nobel, d'abord à l'équipe responsable de sa production (1945, Prix Nobel de médecine), ensuite à la chimiste ayant élucidé sa structure (1964, Prix Nobel de chimie).

Grace aux antibiotiques…

D'importantes avancées dans divers traitements médicaux ont été rendues possibles grâce aux antibiotiques (Marti et al. 2014). Malheureusement, ce succès est actuellement éclipsé par l'augmentation de la fréquence des micro-organismes résistants aux traitements. L'émergence et la multiplication d'organismes résistants à la plupart des agents thérapeutiques couramment utilisés fait planer une menace sur l'avenir de l'humanité, d'autant plus que la mise sur le marché de nouveaux agents thérapeutiques s'est fortement ralentie ces dernières années. La résistance croissante des organismes soulève également la question de leur dissémination et leur destin environnemental; les transferts entre microbiome humain et environnements naturels et réciproquement, et les échanges de gènes entre organismes pathogènes et environnementaux.

> «Un inventaire incluant des marqueurs pour différentes classes d'antibiotiques permettrait de mieux évaluer le réservoir environnemental de gènes de résistance.»

Profiter du passé

L'histoire de la découverte de la pénicilline est un exemple fascinant pour la science. Nous pouvons encore aujourd'hui en tirer de nombreux enseignements: l'importance des découvertes inattendues et celle de la communication avec le grand public dans le domaine scientifique, le rôle des interactions biologiques dans l'émergence de phénomènes complexes, la valeur ajoutée du travail interdisciplinaire, la place centrale de l'écologie chimique dans l'intégration des différents niveaux biologiques, ou encore celle de la sélection naturelle dans l'évolution microbienne, entre autres. Pour toutes ces raisons, la recherche de solutions à la crise actuelle des antibiotiques pourrait bénéficier d'un regard critique et intégratif sur le passé.

La résistance à un antibiotique peut apparaître spontanément, mais il est généralement accepté qu'une telle résistance engendre un coût évolutif important pour l'organisme. Par conséquent, une augmentation de la fréquence des résistances est associée à une forte pression de sélection, par exemple via l'augmentation de l'utilisation d'un antibiotique donné (Bengtsson-Palme et al. 2018). On s'attend par conséquent à une augmentation de la fréquence des gènes de résistance détectés tant dans les milieux hospitaliers que naturels, liée à l'historique d'utilisation. De nombreux indices confirment une telle augmentation dans les milieux hospitaliers, mais ils sont plus rares dans le cas des milieux naturels. Etudier l'effet de l'utilisation des antibiotiques sur le réservoir environnemental de gènes de résistance au cours du temps pose un certain nombre de problèmes méthodologiques. L'étude des communautés bactériennes environnementales inclut des méthodes métagénomiques, c'est-à-dire en analysant le matériel génétique (ADN) extrait d'échantillons environnementaux. Dans le cas d'échantillons anciens, un des problèmes principaux consiste à trouver des échantillons naturels datables suffisamment bien conservés, ayant subi un minimum de modifications/dégradation suite à leur formation. Ces conditions sont remplies par exemple par deux séries de sols récoltés au Pays-Bas et au Danemark sur plusieurs années. Ces sols ont permis de mettre en évidence une corrélation entre l'utilisation passée d'antibiotiques et l'augmentation de la fréquence des gènes de résistance, dans deux études indépendantes (Graham et al. 2016; Knapp et al. 2010).

La recherche aux archives naturelles

Récemment, nous avons utilisé des carottes de sédiments pour démontrer comment ces archives naturelles peuvent contribuer à l'étude passée des résistances aux antibiotiques. Les sédiments se forment par couches successives (dépôt de matière minérale et organique). Ils constituent des archives environnementales chronologiques ans lesquelles s'accumulent des marqueurs chimiques et biologiques qui peuvent par la suite être récupérés et analysés (Thevenon et al. 2012). L'utilisation de sédiments n'est pas sans faille car ces signaux biologiques ne se préservent pas de la même façon pour tous les organismes. Pour cette raison, des études actuelles ciblent l'ADN extrait à partir de structures de survie (spores) produites par certaines

1 Laboratoire de microbiologie, Université de Neuchâtel
2 Vital-IT, Institut suisse de bioinformatique

bactéries, structures résistantes à la dégradation favorisant leur préservation (Wunderlin et al. 2014a; Wunderlin et al. 2016; Wunderlin et al. 2014b). La fréquence de gènes de résistance a été mesurée à partir de l'ADN extrait de ces spores. Avec cette méthode, l'analyse de sédiments du lac Léman, datant de 1920 à 2015, a mis en évidence une corrélation entre l'utilisation médicale de deux antibiotiques (la tétracycline et la sulfonamide) et la fréquence de gènes conférant la résistance à chacun de ces antibiotiques. De plus, un lien entre l'augmentation de la fréquence des gènes de résistance et des changements dans la structure des communautés microbiennes a pu être établi (Madueno et al. 2018). L'augmentation de la fréquence du marqueur *tet*(W), un déterminant conférant la résistance à la tétracycline, a pu être reliée à une augmentation de l'abondance relative des firmicutes, un groupe de bactéries parmi les plus abondantes du microbiome humain (Browne et al. 2016). Un tel lien suggère un fort impact anthropique et soutient la thèse d'un lien entre consommation d'antibiotiques et augmentation du réservoir environnemental de gènes de résistance.

Gardez un œil sur la grande image

Une vision écologique du rôle des antibiotiques nous offre aujourd'hui une représentation plus complexe de ces molécules que comme simples agents d'anéantissement des populations microbiennes indésirables (Taylor et al. 2011). La grande diversité des espèces bactériennes et fongiques les produisant, ainsi que leur implication dans la communication intra- et interspécifique (Goh et al. 2002), suggèrent que ces molécules ont un rôle très important dans l'évolution des communautés microbiennes (Martinez 2008). De nombreuses questions restent encore ouvertes. Un lien a pu être établi entre l'utilisation d'antibiotique en médicine humaine, la fréquence de gènes de résistance, et la structure de la communauté bactérienne, dans un lac subissant une forte pression anthropique. Mais qu'en est-il des milieux naturels moins affectés par les activités humaines? A l'heure actuelle, nos recherches se sont limitées à deux gènes de résistance, mais un inventaire incluant des marqueurs pour différentes classes d'antibiotiques, ou fait à partir de la reconstruction de génomes environnementaux, permettrait de mieux évaluer le réservoir environnemental de gènes de résistance. Finalement, l'impact écologique de ces structures de survie, dont l'activité métabolique est réduite, ainsi que leur rôle dans la dissémination et la transmission des gènes de résistance, restent à déterminer. Ce point devrait être abordé par des expériences de ressuscitation (ou réactivation) et d'évolution en laboratoire.

Correspondance
Pilar.Junier@unine.ch

Sedimentmikrobiota: neuartiger Ansatz zur Rückverfolgung der Antibiotikaeinnahme in der Vergangenheit

Die Entdeckung von Antibiotika und deren Verwendung im Kampf gegen Infektionskrankheiten stellt einen der bedeutendsten Erfolge in der Geschichte der medizinischen Mikrobiologie dar. Bedauerlicherweise hat der teilweise missbräuchliche und gedankenlose Einsatz, insbesondere in der Lebensmittelindustrie und zu präventiven Zwecken, unausweichlich zur Herausbildung resistenter Mikroorganismen geführt. Seit Beginn der Verwendung von Antibiotika hat die Verbindung zwischen Medizin und Natur zu einer «evolutionären Erfahrung» geführt, mit der niemand gerechnet hatte. Eine Untersuchung der Ergebnisse aus dieser Erfahrung der Vergangenheit könnte zur Entwicklung und Umsetzung einer globalen Strategie beitragen, mit der künftig der Einsatz von Antibiotika besser in den Griff zu bekommen ist.

Bibliographie

Bengtsson-Palme J, Kristiansson E, Larsson DGJ (2018) Environmental factors influencing the development and spread of antibiotic resistance. FEMS Microbiol Rev 42(1) doi:10.1093/femsre/fux053
Browne HP, Forster SC, Anonye BO, Kumar N, Neville BA, Stares MD, Goulding D, Lawley TD (2016) Culturing of 'unculturable' human microbiota reveals novel taxa and extensive sporulation. Nature 533(7604):543-6 doi:10.1038/nature17645
Goh EB, Yim G, Tsui W, McClure J, Surette MG, Davies J (2002) Transcriptional modulation of bacterial gene expression by subinhibitory concentrations of antibiotics. Proc Natl Acad Sci U S A 99(26):17025–30 doi:10.1073/pnas.252607699
Graham DW, Knapp CW, Christensen BT, McCluskey S, Dolfing J (2016) Appearance of -lactam Resistance Genes in Agricultural Soils and Clinical Isolates over the 20th Century. Scientific Reports 6:21550 doi:10.1038/srep21550
Knapp CW, Dolfing J, Ehlert PAI, Graham DW (2010) Evidence of Increasing Antibiotic ß-Resistance Gene Abundances in Archived Soils since 1940. Environmental Science & Technology 44(2):580–587 doi:10.1021/es901221x
Madueno L, Paul C, Junier T, Bayrychenko Z, Filippidou S, Beck K, Greub G, Burgmann H, Junier P (2018) A historical legacy of antibiotic utilization on bacterial seed banks in sediments. PeerJ 6:e4197 doi:10.7717/peerj.4197
Marti E, Variatza E, Balcazar JL (2014) The role of aquatic ecosystems as reservoirs of antibiotic resistance. Trends Microbiol 22(1):36–41 doi:10.1016/j.tim.2013.11.001
Martinez JL (2008) Antibiotics and antibiotic resistance genes in natural environments. Science 321(5887):365-7 doi:10.1126/science.1159483
Taylor NG, Verner-Jeffreys DW, Baker-Austin C (2011) Aquatic systems: maintaining, mixing and mobilising antimicrobial resistance? Trends Ecol Evol 26(6):278–84 doi:10.1016/j.tree.2011.03.004
Thevenon F, Adatte T, Wildi W, Pote J (2012) Antibiotic resistant bacteria/genes dissemination in lacustrine sediments highly increased following cultural eutrophication of Lake Geneva (Switzerland). Chemosphere 86(5):468–76 doi:10.1016/j.chemosphere.2011.09.048
Wunderlin T, Corella J, Junier T, Bueche M, Loizeau J-L, Girardclos Sp, Junier P (2014a) Endospore-forming bacteria as new proxies to assess impact of eutrophication in Lake Geneva (Switzerland-France). Aquat Sci 76(1):103–116 doi:10.1007/s00027-013-0329-0
Wunderlin T, Junier T, Paul C, Jeanneret N, Junier P (2016) Physical Isolation of Endospores from Environmental Samples by Targeted Lysis of Vegetative Cells. J Vis Exp(107) doi:10.3791/53411
Wunderlin T, Junier T, Roussel-Delif L, Jeanneret N, Junier P (2014b) Endospore-enriched sequencing approach reveals unprecedented diversity of Firmicutes in sediments. Environ Microbiol Rep 6(6):631-9

Poster Presentations

Poster presentation at Annual PhD Student Meeting from Doctoral School of Organismal Biology, Neuchâtel, Switzerland (March 29, 2017)

ENDOSPORE DNA AS A PROXY TO STUDY THE DISSEMINATION AND ACCUMULATION OF ANTIBIOTIC RESISTANCE GENES IN THE ENVIRONMENT

Christophe Paul[1] , Zhanna Bayrychenko[1], Laura Madueño[1,2], Sevasti Filippidou[1*], Thomas Junier[3], Karin Beck[4], Tina Wunderlin[1], Gilbert Greub[5], Helmut Bürgmann[4], Pilar Junier[1]

1Laboratory of Microbiology, University of Neuchâtel, Neuchâtel, Switzerland
2Centro de Investigación y Desarollo en Fermentaciones Industriales, UNLP-CCT CONICET, La Plata, Argentina.
3Vital-IT group, Swiss Institute of Bioinformatics, Lausanne, Switzerland
4Department of Surface Waters — Research and Management, Eawag, Swiss Federal Institute of Aquatic Science and Technology, Kastanienbaum, Switzerland
5Institute of Microbiology, University Hospital Center, University of Lausanne, Lausanne, Switzerland.

INTRODUCTION

Antibiotic resistance genes (ARGs)

- Extensive use of antibiotics led to the emergence of highly resistant organisms
- Represents a future threat for human health
- Aquatic ecosystems serve as a reservoir of antibiotic resistance genes = Pathway for dissemination of resistant organisms
- Possible co-selection between ARGs and trace metals

No biological marker to establish their origin, spread and evolution over time

Endospores

- Present only in Firmicutes
- Endospores are highly resistant structures
- Resist harsh conditions for extended periods of time
- High potential of dispersion and potentially for the spread of ARGs

Might constitute an ideal biological marker for such studies.

Evaluation of the accumulation and dissemination of ARGs in endospores DNA in a trace metals contaminated aquatic ecosystem.

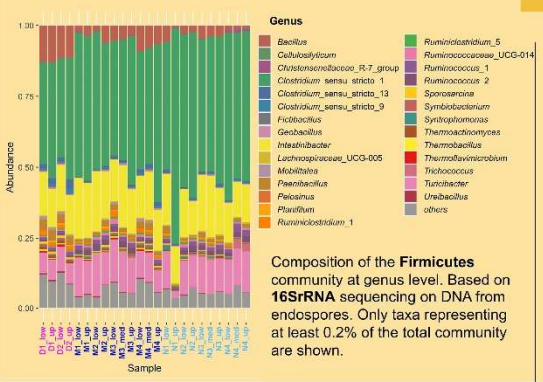
METHODS

- Outlet of wastewater treatment plant, Vidy Bay
- Quantification of two commonly studied ARGs (***tetW*** and ***sul1***) by **qPCR**
- **16SrRNA amplicons sequencing** from endospore DNA
- Correlation between endospores community, ARGs and environmental parameters (Corg, Ntot, Al, As, Cd, Cu, Fe,,Mn, Zn)

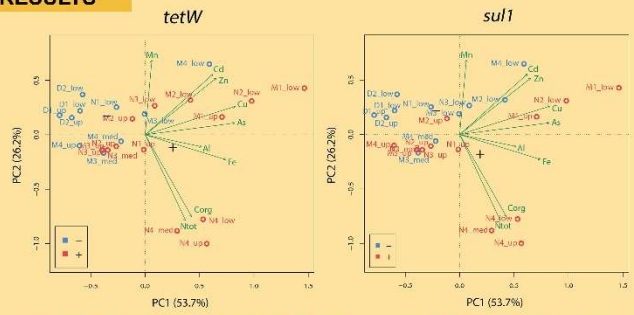
Area divided in 3 zones:
- Near the outlet (N)
- Middle zone (M)
- Distal (D)

Samples retrieved at 2 or 3 depths within sediment cores:
- 0-1.5 cm (up)
- 1.5-3 cm (med)
- 3-9 cm (low)

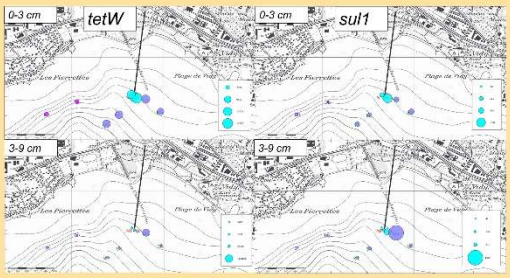
RESULTS



Composition of the **Firmicutes** community at genus level. Based on **16SrRNA** sequencing on DNA from endospores. Only taxa representing at least 0.2% of the total community are shown.



PCA on the environmental parameters. Arrows represent the contribution of variables to the axis. Colors separate samples depending on **ARGs frequency**, above (+) or below (-) the mean concentration. On the left *tetW*, on the right *sul1*.



Spatial distribution of ARGs in the Vidy Bay. On the left *tetW*, on the right *sul1*. Circle size represents ARGs frequency (= normalized concentrations = copies of genes/ng DNA). Circle color represents the zone (blue= Near, violet=Middle, purple=Distal). A positive correlation was found between the normalized concentrations (copies of genes/ng DNA) of ARGs and the distance to the outlet pipe of the WWTP for both genes (only in upper sediment if separated).

CONCLUSIONS (provisional)

- All samples are dominated by the genus ***Clostridium*** => influence of the WWTP even in distal zone
- *Bacillus* is more abundant in samples from the distal zone
- **Different distribution pattern** for both ARGs and layers
- **Linear decrease of ARGs** with the distance to the outlet pipe (in the upper sediment)
- *tetW* detected at higher abundance/frequency than *sul1*
- Both genes frequency correlated to Corg, Ntot and Fe
- ***tetW*** frequency **correlated** to **Cu**, **Al** and **As**

- **WWTP** suspected as the **source of endospores containing ARGs**
- **Endospore DNA** is selectively **enriched in *tetW***
- Endospore-forming bacteria have an **important role in the active selection of certain ARGs**
- Endospores DNA might be **enriched in ARGs in human microbiota**
- **No evidence for co-selection** against co-deposition of ARGs and trace metals

Acknowledgements: FNSNF Fonds national suisse Schweizerischer Nationalfonds Fondo nazionale svizzero Swiss National Science Foundation Grant N: CR23I2_162810

Contact: christophe.paul@unine.ch

Poster presentation at Annual Swiss Conference on Ecology, Evolution, Systematics and Conservation (Biology18), Neuchâtel, Switzerland (February 15-16, 2018) and at Annual Assembly of the Swiss Society of Microbiology (SSM), Lausanne, Switzerland (August 28-30, 2018)

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

Christophe Paul[1], Laura Madueño[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, University of Neuchâtel, Neuchâtel, Switzerland
[2] Vital-IT group, Swiss Institute of Bioinformatics, Lausanne, Switzerland
[3] Department of Surface Waters — Research and Management, Eawag, Swiss Federal Institute of Aquatic Science and Technology, Kastanienbaum, Switzerland
[4] Institute of Microbiology, University Hospital Center, University of Lausanne, Lausanne, Switzerland.

Introduction

Antibiotic resistance genes (ARGs)

- **Extensive use** of antibiotics has led to the emergence of **highly resistant** organisms
- Represents a future **threat for human health**
- **Aquatic ecosystems** have an **outlet function** and serve as a **reservoir** of antibiotic resistance genes
- **No biological marker** to establish their accumulation and persistence over time

What is the seed bank?

- **Spore-like structures** = resistant forms
- **Dormancy** state = low metabolism activity (inactive)
- **Reversible** state = sporulation vs reactivation
- **Common in bacteria** (Actinobacteria, Myxobacteria, Cyanobacteria and Firmicutes)
- Prevalent **in human/animal microbiome**

Why studying ARGs in seed bank?

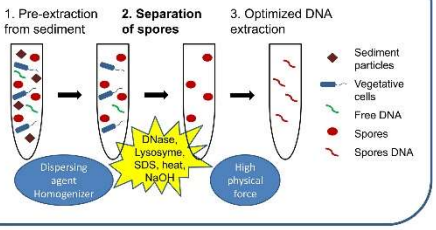
- **Preserved** in sediments
  - historical accumulation
  - long-term ARG pool
- **Dispersal** capacity increases…
  - ARG distribution
  - mixing with environmental strains
  - risk of reintroduction

Bacterial seed bank might constitute an ideal biological marker for tracking ARGs in the environment

Methods

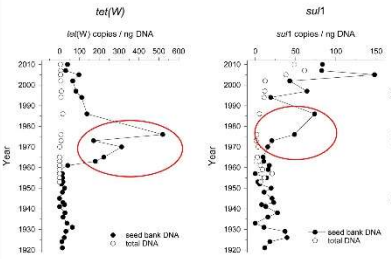
- Inactive canyon in Rhone delta, **Lake Geneva** (red circle)
- **Sediment core** (1 meter long representing **~100 years**)
- **Quantification** of two ARGs (***tet(W)*** and ***sul1***) by **qPCR**
- **16S rRNA gene** amplicon **sequencing** of seed bank
- **Correlation analyses** between seed bank community and ARGs

How to study ARGs in seed bank DNA?



Results

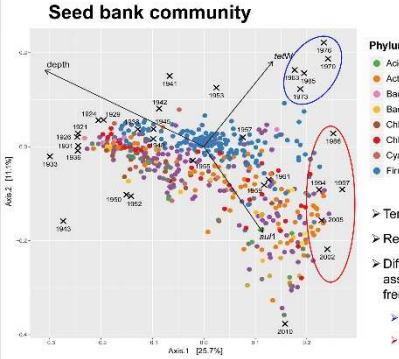
ARG accumulation over time



- Higher ARG abundance in seed bank DNA
- Increasing levels of ARGs from 1960 (*tet(W)*) and 1970 (*sul1*)
- Different accumulation patterns for *tet(W)* and *sul1*

Relative abundance of *tet(W)* and *sul1* in DNA extracted from the seed bank and the total microbial community, between 1920 and 2010.

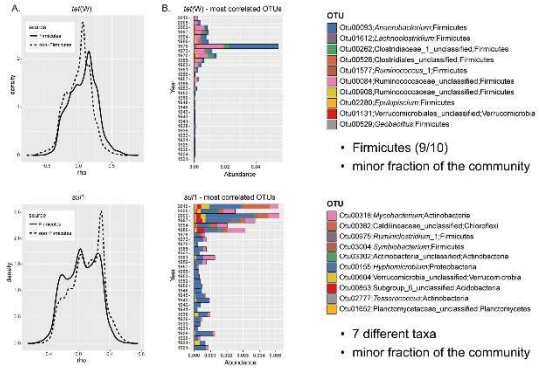
Seed bank community



- Temporal gradient (depth)
- Related to lake eutrofication
- Different communities associated to *tet(W)* or *sul1* frequency:
  - *tet(W)* -> Firmicutes
  - *sul1* -> diverse taxa

Principal coordinates analysis (PCoA) of the seed bank bacterial community showing the effect of lake eutrophication (Axis 1; vector depth) and the accumulation of ARG (vector *tetW* and *sul1*; circles blue and red respectively)

ARGs vs OTUs correlations



- Firmicutes (9/10)
- minor fraction of the community

- 7 different taxa
- minor fraction of the community

(A) Kernel density curves representing the frequency of Spearman correlation coefficients calculated for each OTU and ARG, plotted as a continuum. (B) Relative abundance of the 10 most positively correlated OTUs for each ARG.

Conclusions

- **Seed bank DNA enriched in ARGs** compare to total DNA
- **Different accumulation patterns** for *tet(W)* and *sul1*
  - **Increasing ARG abundance** from 1960 (*tet(W)*) and 1970 (*sul1*)
  - **Reflect the temporal use** of their related antibiotic
- **Only few OTUs possess ARGs** → More OTUs possess *sul1*
- **Link** between seed bank **taxonomy and accumulation of specific ARGs:**
  - ***tet(W)*** accumulation highly correlated with changes in the abundance of **Firmicutes**
  - ***sul1*** accumulation correlated with a **wide range of taxonomic groups**

Seed bank can be used to study the historical usage of antibiotics and resistance prevalence

Acknowledgements: FNSNF Fonds National Suisse Schweizerischer Nationalfonds Fondo Nazionale Svizzero Swiss National Science Foundation Grant N: CR23I2_162810

Contact: christophe.paul@unine.ch

Poster presentation at 7th Swiss Microbial Ecology Meeting (SME), Lausanne, Switzerland. January 31-February 1, 2019

TO SPORE OR NOT TO SPORE: AN INSIGHT INTO THE ENVIRONMENTAL SPOROBIOTA

Christophe Paul[1], Thomas Junier[1,2], Sevasti Filippidou[1], Mathilda Hayoz[1], Anael Lehmann[3], Patrick S. Chain[4], Daniel Ariztegui[5], Torsten Vennemann[3], Patrick H. Viollier[6], Pilar Junier[1]

[1] Laboratory of Microbiology, University of Neuchâtel, Neuchâtel, Switzerland
[2] Vital-IT group, Swiss Institute of Bioinformatics, Lausanne, Switzerland
[3] Laboratory of stable isotope geochemistry. Institute of Earth Surface Dynamics, University of Lausanne, Lausanne, Switzerland
[4] Bioscience Division, Los Alamos National Laboratory, Los Alamos, New Mexico, USA
[5] Laboratory of Limnogeology and Geomicrobiology, Department of Earth Sciences, University of Geneva, Geneva, Switzerland
[6] Department of Microbiology and Molecular Medicine, Faculty of Medicine, University of Geneva, Geneva, Switzerland

INTRODUCTION

What are spores?

- **Specialized cellular structure** = resistant forms
- **Dormant** state = inactive (low metabolism activity) and reversible
- High potential for **dispersal**
- Withstand **harsh** (unfavorable) **conditions**

Persistence + Dissemination => Successful survival strategy

Diversity and distribution of spores

- **Common** in diverse bacterial phyla: **Firmicutes** (endospores), **Actinobacteria** (exospores), **Myxobacteria** (myxospores/fruiting bodies), **Cyanobacteria** (akinetes)
- Distribution and prevalence widely unknown
- High potential for **dispersal** (inactive thermophiles found in cold marine sediment)

=> Is everything everywhere???

An organism is considered as sporulant only if spores has been observed
Prediction mainly based on morphological and genetic information

What is the real diversity, distribution and prevalence of spore formers???

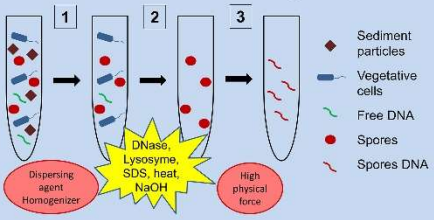
METHODS

Sampling and analyses

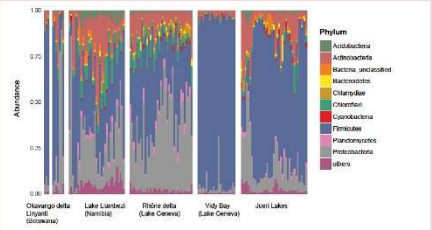
- **3 geographical regions:**
  - **Botswana/Namibia** (Lake Liambezi, Linyanti, Okavango delta)
  - **Lake Geneva (CH)** (Vidy Bay (WWTP), Rhône delta)
  - **Joeri lakes (CH)** = alpine lakes
- **Sediment , soil** and **water** samples
- **Separation of spores** from vegetative cells
- **16S rRNA gene** amplicon **sequencing** of putative spores (lysis-resistant cells)
- Analysis of the **lysis-resistant bacterial community**

1. **Pre-extraction of cells** from sediment particles using dispersing agent (1% Na-hexametaphosphate) and homogenizer (Ultra-Thurrax).
2. **Physical isolation of «spores»** from vegetative cells by physicochemical treatment (heat, lysozyme and a mix of NaOH and SDS).
3. **Optimized DNA extraction protocol** using high physical force (= 3 sequential bead beating steps).

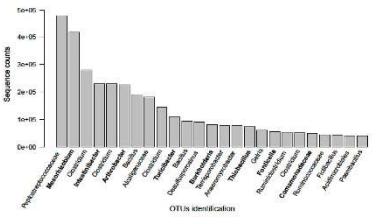
Indirect DNA extraction in 3 steps:



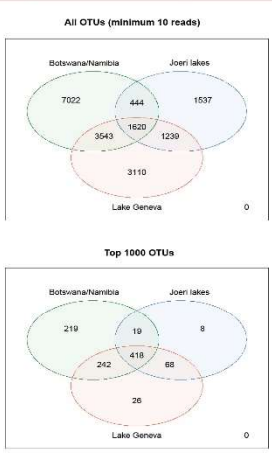
RESULTS



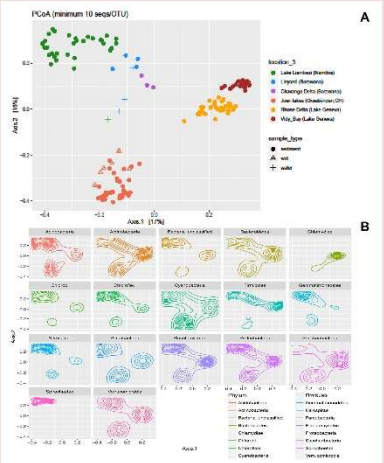
Composition of the environmental lysis-resistant bacterial community, at phylum level, based on 16S rRNA gene sequencing. Relative abundance of the most abundant phyla (> 0.5%).



Total number of reads of the 25 most abundant lysis-resistant OTUs, classified at genus level. In bold, genera in which spore-formation has not been reported.



Venn diagrams showing the number of shared OTUs between the three sampling locations. On top all OTUs (minimum 10 reads) were considered (18'515 OTUs). On the bottom only the 1000 most abundant OTUs were considered.



Principal coordinates analysis (PCoA) of the lysis-resistant bacterial community showing (A) the variability of the community among sites, (B) and the density distribution of OTUs for the most abundant phyla (OTUs with minimum 10 reads and phyla over 0.5% mean relative abundance in the entire dataset).

CONCLUSIONS

- **3 highly enriched phyla**: **Firmicutes**, **Proteobacteria** and **Actinobacteria**, all known for the production of spores.
- Many **genera not known to sporulate** or to form an equivalent specialized lysis-resistant cell structure.
- Most OTUs have a **site-specific distribution**, although most phyla are cosmopolitans.
- Some **diagnostic taxa** (ex. Chlamidiae for a human-impacted environment (Lake Geneva), Spirochaetae for Botswana/Namibia).
- Distribution of OTUs among the 3 environments supports the hypothesis of a **relationship between species abundance and dispersal potential**.

The production of a durable cell structure might be a widespread adaptation of environmental bacteria

Acknowledgements: FNSNF Fonds National Suisse Schweizerischer Nationalfonds Fondo Nazionale Svizzero Swiss National Science Foundation Grant N: CR23I2_162810

Contact: christophe.paul@unine.ch

Curriculum Vitae

Christophe Paul

6th April 1980
Swiss, 1 child
Port-Roulant 44
CH-2000 Neuchâtel

Mob: 076 472 20 54
Tel: 032 730 53 46
E-mail: christophe.paul@unine.ch

EDUCATION - DEGREES

Date	2015 - now
Title awarded	**PhD in Geomicrobiology** (directed by Prof. Pilar Junier)
Place	Laboratory of microbiology, Institute of biology, University of Neuchâtel (CH)
Subject	*Spore-forming bacteria as a proxy for the reconstruction of past environment and possible use for the detection of antibiotic resistance genes in the environment.*

Date	2007 - 2011
Title awarded	**Master of Science in Biogeoscience** (with high distinction), Master thesis in microbial ecology
Place	Institute of biology, University of Neuchâtel (CH)
Thesis subject	*Biochemical cycle of iron and seasonality of bacterial communities in Lake Loclat* (directed by Dr J. Zopfi).

Date	2004 - 2007
Title awarded	**Bachelor of Science in Biology**
Place	Institute of biology, University of Neuchâtel (CH)

PROFESSIONAL EXPERIENCE

Date	11/2014 - 04/2015
Position	**Scientific collaborator in microbiology**
Place	Laboratory of microbiology, Institute of biology, University of Neuchâtel (CH)
Activities	Use of endospores as a new environmental proxy in paleoecology, detection and quantification of antibiotic resistance genes in natural environments.

Date	06/2014 - 09/2014 and 04/2011 - 12/2012
Position	**Laboratory technician/Scientific collaborator in microbiology**
Place	Laboratory of soil biology, Institute of biology, University of Neuchâtel (CH)
Activities	Identification of Phytophthora spp. from soil samples using NGS and bioinformatics tools, identification and clonality of Saprolegnia parasitica pathogenic of the Doubs fish populations, Phylogeny/barcoding of oomycetes, writing of publications.

Date	09/2008 - 09/2009
Position	**Field assistant**
Place	Laboratory of microbiology, Institute of biology, University of Neuchâtel (CH)
Activities	Sampling, in/ex situ incubations, physico-chemical analyses.

TEACHING RECORD

2015 - 2018	**Supervision** of interns and master students
2017 - 2018	Practical course «**Molecular ecology technics**» (master) and «**Molecular biology**» (bachelor 3rd year)
2016	Practical course «**General bacteriology**» (bachelor 2nd year)
2015	Practical course «**Molecular ecology technics**» (master) and «**Method in biochemistry and molecular biology**» (bachelor 3rd year)
2009	Practical course «**General microbiology**» (propaedeutic 1st year) and «**Geomicrobiology**» (master)

AWARD

08/2018	**Award** (500 CHF) for the 3rd best poster at the Annual Assembly of the Swiss Society of Microbiology (SSM), Lausanne (CH)

PUBLICATIONS

2019	Hashmi I., Paul C., Al-Dourobi A., Sandoz F., Deschamps P., Junier T., Junier P., Bindschedler S. 2019. Comparison of the plant growth promotion performance of a consortium of Bacilli inoculated as endospores or as vegetative cells. FEMS Microbiology Ecology. DOI: 10.1093/femsec/fiz147.
2019	Paul C., Filippidou S., Jamil I., Kooli W., House GL., Estoppey A., Hayoz M., Junier T., Palmieri F., Wunderlin T., Lehmann A., Bindschedler S., Vennemann T., Chain PSG., Junier P. 2019. *Bacterial spores, from ecology to biotechnology*. Elsevier Inc. DOI: 10.1016/bs.aambs.2018.10.002.
2018	Paul C., Bayrychenko Z., Junier T., Filippidou S., Beck K., Bueche M., Greub G., Bürgmann H., Junier P. 2018. Dissemination of antibiotic resistance genes associated with the sporobiota in sediments impacted by wastewater. PeerJ 6:e4989. DOI: 10.7717/peerj.4989.
2018	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. DOI: 10.7717/peerj.4197.
2016	Wunderlin T., Junier T., Paul C., Jeanneret N., Junier P. 2016. Physical Isolation of Endospores from Environmental Samples by Targeted Lysis of Vegetative Cells. JOVE-Journal of Visualized Experiments. DOI: 10.3791/53411.
2014	Steciow MM., Lara E., Paul C., Pillonel A., Belbahri L. 2014. Multiple barcode assessment within the Saprolegnia-Achlya clade (Saprolegniales, Oomycota, Straminipila) brings order in a neglected group of pathogens. IMA Fungus 5:439–448. DOI: 10.5598/imafungus.2014.05.02.08.