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Heavy metal pollution and co-selection for antibiotic resistance: A microbial palaeontology approach

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ABSTRACT

Frequent and persistent heavy metal pollution has profound effects on the composition and activity of microbial communities. Heavy metals select for metal resistance but can also co-select for resistance to antibiotics, which is a global health concern. We here document metal concentration, metal resistance and antibiotic resistance along a sediment archive from a pond in the North West of the United Kingdom covering over a century of anthropogenic pollution. We specifically focus on zinc, as it is a ubiquitous and toxic metal contaminant known to co-select for antibiotic resistance, to assess the impact of temporal variation in heavy metal pollution on microbial community diversity and to quantify the selection effects of differential heavy metal exposure on antibiotic resistance. Zinc concentration and bioavailability was found to vary over the core, likely reflecting increased industrialisation around the middle of the 20th century. Zinc concentration had a significant effect on bacterial community composition, as revealed by a positive correlation between the level of zinc tolerance in culturable bacteria and zinc concentration. The proportion of zinc resistant isolates was also positively correlated with resistance to three clinically relevant antibiotics (oxacillin, cefotaxime and trimethoprim). The abundance of the class 1 integron-integrase gene, *intI1*, marker for anthropogenic pollutants correlated with the prevalence of zinc- and cefotaxime resistance but not with oxacillin and trimethoprim resistance. Our microbial palaeontology approach reveals that metal-contaminated sediments from depths that pre-date the use of antibiotics were enriched in antibiotic resistant bacteria, demonstrating the pervasive effects of metal-antibiotic co-selection in the environment.

1. Introduction

The global emergence of antimicrobial resistance (AMR) is of acute concern: a 2014 review on tackling this crisis estimated the number of deaths that may occur globally due to AMR in the next 35 years could be as high as 300 million individuals and that the economic damage could amount to 60–100 trillion US dollars (O'neill, 2014). While the misuse of antimicrobials in human medicine and agricultural practices has received widespread attention, the role of the natural environment in the dissemination of- and selection for AMR is increasingly appreciated (Wellington et al., 2013). For instance, antibiotics, biocides and resistant bacteria can enter the environment via point sources (e.g. sewage) and nonpoint sources (e.g. agricultural runoff) pollution (Alonso et al., 2001). Pollution with heavy metals also has the potential to result in the spread of AMR (Baker-Austin et al., 2006). Bacteria are generally highly sensitive to metal pollution but can evolve a variety of resistance mechanisms, mediated by chromosomal mutations or by the uptake of resistance genes on mobile genetic elements (MGEs) (Giller

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et al., 1998; Bruins et al., 2000). When a genetic change mediates resistance to both metals and antibiotics (cross-resistance), or when metal resistance- and antibiotic resistance genes are genetically linked on MGEs (co-resistance), metals can co-select for resistance to clinically relevant antibiotics (Baker-Austin et al., 2006; Peltier et al., 2010; Seiler and Berendonk, 2012).

Although heavy metals occur naturally, they are increasingly prevalent due to anthropogenic activities such as mining, industry, burning of fossil fuels and agricultural practices (Valette-Silver, 1993; Nicholson et al., 2003; François et al., 2012). Heavy metals can be toxic at relatively low concentrations and persist for long periods, and are therefore considered a significant environmental issue and a threat to public health (Peltier et al., 2010; Kawane, 2012; Samanta et al., 2012; Garhwal et al., 2014; Laghlimi et al., 2015; Hussey et al., 2017). Anthropogenic heavy metal pollution can affect the biomass (Samanta et al., 2012), diversity (Sobolev and Begonia, 2008; Berg et al., 2012) and metabolic activity (Hoostal et al., 2008) of resident microbial communities. Positive correlations between the level of heavy metals in soils and sediments and the occurrence of metal resistance or stress-related genes have been observed in a range of studies (Bouskill et al., 2007; Seiler and Berendonk, 2012; Xiao-Fang et al., 2012; Besaury et al., 2013; Zhao et al., 2019), and metal-contaminated sites have been found to contain elevated levels of antibiotic resistant organisms (Wright et al., 2006; Berg et al., 2010).

The effects of metal pollution on antibiotic resistance have been uncovered using diverse approaches, including cultivation in microcosms enriched with defined metal concentrations (Stepanauskas et al., 2006), correlating metal concentrations with the presence of antibiotic resistance genes in environmental samples (Knapp et al., 2011; Zhao et al., 2019), quantifying resistance to metals and antibiotics in clones isolated from metal-amended field plots (Berg et al., 2005) or from livestock fed with metal-supplemented feed (Bednorz et al., 2013; Yazdankhah et al., 2014; Zhao et al., 2019) or analysing genetic linkage of metal- and antibiotic resistance genes in bacterial genomes using bioinformatics methods (Pal et al., 2015). An approach that has received relatively little attention is the quantification of metal and antibiotic co-selection within sedimentary archives.

Taking a temporal, rather than a spatial or experimental approach has the advantage that the distinct timescales of historical metal- and antibiotic pollution can be separated, allowing the untangling of selection and co-selection for resistance. Concentrations of heavy metals and other pollutants have been successfully quantified from lake sediment records (Audry et al., 2004), including their effects on microbial community composition (Kaci et al., 2016). Konstantinidis and colleagues isolated copper resistant bacteria from lake sediments highly polluted with mining waste and showed that isolates from older, deeper sediments with higher copper concentrations had higher levels of resistance to copper (Konstantinidis et al., 2003). Most studies on the temporal effects of metal pollution on microbial community structure and resistance as archived in sediments have focused on a relatively limited range of recent depths and ages and characterise the sediment composition without establishing quantitative data on metal contamination (Bouskill et al., 2007; Kaci et al., 2016).

In this study, we took a correlative 'microbial palaeontology' approach to quantify temporal variation in metal pollution throughout an urban sediment core to relate this to both metal and antibiotic resistance of the resident bacterial community quantified via both culture-dependent and independent methods. We focused on sediment from a pond (Griffin Wood Pond; GWP) located directly down prevailing wind of major historical and present-day chemicals manufacturing centres in the north-west of the U.K. (Hardie, 1950; Jones, 1969). A depositional history of atmospheric metal pollution was reconstructed by radiometric dating and measuring metal concentrations down the core. Zinc is among the most common and toxic heavy metals found in polluted sites (Choudhury and Srivastava, 2001a, 2001b, Mansoorian et al., 2014), including our study site, and therefore was used as our focal metal contaminant. In addition to measuring total zinc concentration we utilised a biosensor to quantify zinc bioavailability down the core.

We used culture-independent methods to quantify the impact of metal pollution on microbial community composition using 16S rRNA amplicon sequencing. We furthermore used qPCR to quantify the class one integron-integrase gene *intI1* in the sediment archive. *IntI1* is commonly linked to genes conferring resistance to heavy metals, biocides and antibiotics and therefore has been used as a genetic marker for anthropogenic pollution (Partridge et al., 2001; Baker-Austin et al., 2006; Hegstad et al., 2010; Gillings et al., 2015), and has been found to be enriched at metal polluted sites (Stokes et al., 2006; Wright et al., 2006; Gillings et al., 2008; Rosewarne et al., 2010; Seiler and Berendonk, 2012). The location of *intI1* gene has had a previously reported proximity to genes coding for efflux based resistance to Zn^{2+} specify strain (Stokes et al., 2006; Gillings et al., 2008), which is a known mechanism of resistance to β-lactams.

By isolating bacteria from different depths along the sediment core, impacted differentially by zinc contamination, we were able to directly associate historical zinc pollution with both zinc resistance and resistance to three clinically important antibiotics. Cefotaxime is a third-generation cephalosporin (Fani et al., 2013) that is used in the treatment of sexually transmitted infections (Golparian et al., 2014; van Dam et al., 2014). Oxacillin is a broad-spectrum β-lactam antibiotic, resistance to which has emerged rapidly over the last several years (Phitaktim et al., 2016). Trimethoprim is a synthetic antimicrobial that is widely applied due its low cost of production and its efficacy (Ho and Juurlink, 2011). Our approach revealed that metal-contaminated sediments from depths that pre-date the use of antibiotics were enriched in antibiotic resistant bacteria, demonstrating the pervasive effects of metal-antibiotic co-selection (Fig. 1).

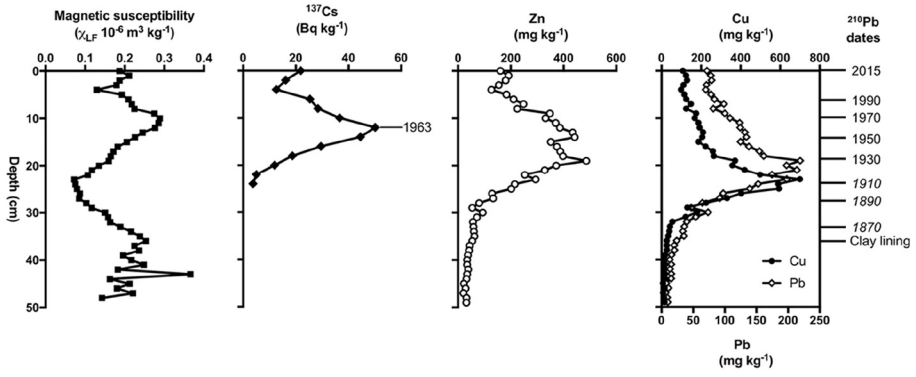

Fig. 1. Down-core concentrations in magnetic susceptibility, ^{137}Cs, Zn, Pb and Cu in Griffin Wood Pond sediments. Corresponding ^{210}Pb dates are shown with depth (italicised dates are extrapolated from the ^{210}Pb chronology). Also highlighted is the ^{137}Cs concentration maximum at 12–13 cm corresponding to the 1963 fallout maximum from the atmospheric testing of nuclear weapons (Fig. S3).

2. Materials and methods

2.1. Sediment core sampling and processing

A sediment core was extracted from Griffin Wood Pond (National Grid Reference: SJ 53709 90958, Fig. S1) approximately 7 km east of Widnes and Runcorn (Merseyside, U.K.), using an Uwitech sediment corer [Uwitec, Mondsee, Austria] deployed from a small inflatable boat into the centre of the pond site (30 April 2014). GWP is approximately 276 m^2 with a maximum water depth of 1 m. GWP has existed since at least 1890 and historic maps reveal minimal modification to the pond margins and the surrounding catchment since this time (Supplementary methodology). Manufacturing of, among others, car paints containing zinc additives, occurred from 1910 to 60 (Fox et al., 1999) and Imperial Chemicals Industry in Runcorn and Widnes was established in 1926 (Campbell, 1971). Throughout the 20th century, the region experienced industrial diversification and expansion with oil refineries and petrochemicals operational in Ellesmere Port post-1920, increased demands on industry in the region during WWII, increased urbanisation, domestic coal burning, and the operation (from 1951 to 1990) of a nearby coal fired power station 2.5 km north of GWP (Halton Borough Council, 2003; Carter, 1964). Potential sources of metal pollution persist, including glass manufacturing within the adjacent town of St Helens (5 km north west), coal-fired power generation at Warrington (4.6 km south), chemical industries within the nearby urban centres of Runcorn (8.3 km south west) and Widnes (5.5 km south west) and oil refinery operations at Ellesmere Port, (16 km south west) (Hodgson et al., 2004) (Fig. S1).

The core was transported the following day to the University of Exeter Streatham Campus (Exeter, UK) where it was stored at 5 °C. On 12 February 2015, the core was vertically extruded and cut at 1 cm intervals to a total of 50 cm depth to yield 50 samples. Three ~0.5 g samples were isolated from the centre of each sediment slice under sterile conditions. Two samples were suspended in 600 µl 20% glycerol solution and stored at −20 °C and −80 °C for bacterial isolation. One sample was stored at −80 °C without the addition of glycerol for DNA extraction and bioavailability analysis. The remaining bulk sediment from each layer sample was frozen overnight at −20 °C, after which sediments were freeze dried at −48 °C and < 0.1 bar (ModulyoD Freeze Dryer, Thermo Electron Corp.) for geomagnetic, geochemical and radiometric analysis.

2.2. Magnetic susceptibility

Low frequency (0.46 kHz) magnetic susceptibility was measured using a Bartington MS2B sensor and MS2 meter to determine down-core concentrations in ferrimagnetic grains (Walden et al., 1999). χ_{LF} is also a proxy for atmospheric pollution (Petrovský et al., 2000) and total metal deposition (Yang et al., 2010). Samples were analysed for two cores extracted from GWP to determine the reproducibility of the sediment record (Charlesworth and Lees, 2001).

2.3. Radiometric dating

Dried sediment samples were analysed for ^{210}Pb, ^{226}Ra, and ^{137}Cs by direct gamma assay using Ortec HPGe GWL series well-type coaxial low background intrinsic germanium detectors (Appleby et al., 1986) at the Environmental Radioactivity Research Centre, University of Liverpool, UK. ^{210}Pb was determined via its gamma emissions at 46.5 keV, and ^{226}Ra by the 295 keV and 352 keV γ-rays emitted by its daughter isotope ^{214}Pb following 3 weeks storage in sealed containers to allow radioactive equilibration. ^{137}Cs was measured by its emissions at 662 keV. The absolute efficiencies of the detectors were determined using calibrated sources and sediment samples of known activity. Corrections were made for the effect of self-absorption of low energy γ-rays within the sample (Appleby et al., 1992). ^{210}Pb dates were calculated using the constant rate of supply model (Appleby and Oldfield, 1978) and corroborated using chronostratigraphic markers determined from the ^{137}Cs record. ^{137}Cs is an artificial radionuclide and peak concentrations indicate the maximum atmospheric fall out from nuclear weapons testing in 1963.

2.4. Inductively coupled plasma optical emission spectroscopy analysis

Approximately 0.25 g of dried sample for each 1-cm layer was digested in 3 ml concentrated (70%) HNO_3 in a 100 ml beaker and stirred with a glass rod. Each sample was then placed on a hot plate until dry. A further 3 ml concentrated nitric acid and 0.5 ml hydrochloric acid (37%) were added and samples were heated until brown nitrogen dioxide fumes were observed and allowed to cool to room temperature. The remaining solution and sediment was filtered (Whatman grade 42 Filters) into a 25 ml flask and topped up to a total of 25 ml with deionized water (Melaku et al., 2005). Samples were analysed using Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) using a Varian VISTA-MPX CCD Simultaneous ICP-OES at the Analytical Instrument Laboratory, University of Exeter, to quantify 17 elements present throughout the sediment core. Results were calibrated using a range of dilution strengths of standard solutions (1000 mg/l of CertiPUR mix containing Ag, Al, B, Ba, Bi, Ca, Cd, Co, Cr, Cu, Fe, In, K, Li, Mg, Na, Mn, Pb, Sr, Tl and Zn; Aristar 456262B containing Ti; and Aristar 456102J containing Ta). Technical replicates (10% of total sample number) and quality control samples consisting of defined metal concentrations (4 ppm) were also performed.

2.5. Zinc bioavailability

The Zn specific biosensor *Pseudomonas putida* strain KT2440.2431(pDNPcadA1lux) (Hynninen et al., 2010) was used to determine Zn bioavailability. To correct for matrix effects and non-specific toxicity, *P. fluorescens* DF57-40E7 was used to determine inhibition of the bioluminescence reaction according to the procedure described in Brandt et al. (2008). Strains were grown and harvested as described in Hynninen et al. (2010). *P. putida* cell suspensions were made in a medium containing 40 mM 3-N-morpholinopropanesulfonic acid (MOPS, pH 7.2), 50 mM KCl, 10 mM NH_3Cl, 0,5 mM $MgSO_4$, 0,4% glucose, 1 mM glycerol-2-PO_4, 1 µM $FeCl_3$ and 12 µg ml^{-1} tetracycline, obtaining an OD_{600} = 0.015–0.020. The bioreporter cells were suspended in the medium, and then mixed in a 1:1 ratio with standards (for standard curve calibration) or samples for analysis in a 96-well microtiter plate. Standard solutions covering a range of 0–12.8 µM $ZnSO_4$ were prepared in Milli-Q water. Samples were extracted with Milli-Q water in a sediment (wet weight)-water ratio of 1:5. Microtiter plates were subsequently incubated for 3.5 h for *P. putida* KT2440.2431(pDNPcadA1lux) and bioluminescence was measured using a Fluostar Optima plate reader (BMG Labtech, Offenburg, Germany). The bioavailable Zn concentrations were determined by fitting third order polynomial calibration curves from the biosensor response to the standard solutions using Microsoft Excel 2016.

2.6. 16S rRNA gene PCR amplification and sequencing

DNA for 16S rRNA gene amplicon sequencing was extracted from sediment samples (depth range 11–35 cm) using the FastDNA™ SPIN Kit for Soil (MP Biomedicals, LLC. 29,525 Fountain Pkwy. Solon, OH 44139) using the standard protocol. DNA was quantified (≥ 5 µg $µl^{-1}$) using Qubit 2.0 Fluorometer (Invitrogen, Thermo-Fisher Scientific, Bishop Meadow Road, Loughborough, UK). Agarose gel electrophoresis was used to verify DNA quality, ensuring high molecular weight and minimal degradation. 100 ml 0.8% agarose gels (Thermo-Fisher Scientific, Bishop Meadow Road, Loughborough, UK) with the addition of 5 µl ethidium bromide (Thermo-Fisher Scientific, Bishop Meadow Road, Loughborough, UK) were run at 120 V for 40 min.

Microbial community profiling was performed using Illumina® HiSeq 2500 using SBS Rapid reagents v2 using Hp10, HP11 and HP12 sequencing primers (Illumina Inc.). The primer sequences used for amplification of the 16S rRNA genes V3-V4 region were 341F Amplicon PCR forward primer (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCaG-3′) and 785R Amplicon PCR reverse primer (5′ TCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATcC-3′) (Thijs et al., 2017). These primers have a phosphorothiate oligo (PTO) indicated by the lowercase at the 3′ end to limit endonuclease degradation. Primers used for a second PCR addition of Illumina NexteraXT barcodes and flowcell binding sequence were; P1 Nextera (i5) index primer: 5′-AATGATACGGCGACCACCGAGATCTACAC[i5]TCGTCGGCAGCGTC-3′ and P2 Nextera (I7) indexing primer: 5′-CAAGCAGAAGACGGCATACGAGA[i7]GTCTCGTGGGCTCGG-3′ (Nextera XT DNA Library Prep Kit, Illumina®).

DNA sequencing reads were assembled using Pandaseq (2.3, RDP extended version 1.0.3) (Masella et al., 2012); filtering and primer removal was performed using RDP Tools v2.0.2; chimera removal was performed with USearch v8.1.1861 (Edgar, 2010) using the RDP gold database; sequencing alignment was performed using Infernal v.1.1.1 (Nawrocki and Eddy, 2013); clustering and classifying was performed using RDP Tool v2.0.2 from database: RDP Release 11, Update 4. Sequencing data set deposited with the European Bioinformatics institute (EBI) under the accession number PRJEB33297.

2.7. Zinc resistance and antibiotic resistance

Tryptic Soy Agar (TSA) (Sigma-Aldrich Co. St. Louis, MO, USA) was amended with antibiotics oxacillin, trimethoprim (Sigma-Aldrich Co. St. Louis, MO, USA) and cefotaxime (Molekula Limited, Newcastle Upon Tyne, UK) or $ZnSO_4{\cdot}7H_2O$ (Sigma-Aldrich Co. St. Louis, MO, USA). M9 ×10 buffer was made using KH_2PO_4 (30 g l^{-1}), Na_2HPO_4 (60 g l^{-1}) (Sigma-Aldrich Co. St. Louis, MO, USA) and NaCl (50 g l^{-1}) (Thermo-Fisher Scientific, Bishop Meadow Road, Loughborough, UK). To isolate individual clones, ~25 mg sediment from the glycerol stock solution for each of layers 11–35 was spread onto unamended TSA plates. A total of 96 clones were isolated for each sediment layer using a sterile toothpick and each clone was re-suspended into 50 µl M9 buffer solution in 96-well plate wells (Thermo-Fisher Scientific, Bishop Meadow Road, Loughborough, UK). Clones were then transferred onto TSA plates (control), TSA plates amended with $ZnSO_4{\cdot}7H_2O$ (0.625 mg ml^{-1}), oxacillin (50 µg ml^{-1}), cefotaxime (50 µg ml^{-1}) and trimethoprim (60 µg ml^{-1}) using a 96-pin replicator (Boekel, Fisher Scientific). All plates were incubated at 28 °C for 48 h and bacterial growth scored as present or absent.

2.8. IntI1 qPCR analysis

DNA for qPCR was extracted from sediment samples from depths 11 cm to 35 cm using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, 2746 Loker Ave West, Carlsbad, CA 92010) using the standard protocol. qPCR assays for *intI1* and bacterial 16S rRNA gene fragments were performed in 96-well plates using TaqMan Environmental Master Mix 2.0 (Life Technologies Corp., Applied Biosystems, Woolston, Warrington, UK) designed to offer accurate detection in the presence of a high level of PCR inhibitors (Czekalski et al., 2014). Standard curves were prepared using serial 10-fold dilutions of plasmid DNA containing the respective target gene in a range of 1×10^6 to 10 gene copies. Each 20 µl of reaction mixture consisted of an 18 µl mix of TaqMan Master Mix, primers, probe, Bovine Serum Albumin (BSA) and Dimethyl Sulfoxide (DMSO) and 2 µl of extracted DNA. Primers (Table S1) (Integrated DNA Technologies, Inc., Leuven, Belgium), Probes (Table S1) (Life Technologies Corp. Woolston, Warrington, UK), BSA, DMSO and nuclease-free water (Thermo-Fisher Scientific, Bishop Meadow Road, Loughborough, UK). Assays were performed in duplicate for each DNA extract, standard and negative controls using an Applied Biosystems StepOnePlus real-time PCR system (Applied Biosystems, Woolston, Warrington, UK). *IntI1* prevalence was calculated as abundance relative to the 16S rRNA marker gene.

2.9. Statistical analysis

Pearson's product-moment correlation, linear regression analyses and images were created using RStudio v1.2.1 (Team, 2013), MacQiime Version 1.9.1 20150604 OS10.7 and in some cases edited using GNU Image Manipulation Program 2.8.18. MacQiime Version 1.9.1 20150604 OS10.7 was used in the following analyses: beta-diversity using Bray-Curtis dissimilarity index, Principal Coordinate Analysis plots created using Emperor through Qiime; Mantel test (Navas-Molina, Peralta-Sánchez et al. 2013).

3. Results

3.1. A temporal gradient of metal contamination in an industrially polluted pond sediment archive

A sediment core was extracted from Griffin Wood Pond (GWP), located in the highly industrialised urban landscape of Merseyside in the North West of England which has been a major site of chemical manufacturing for over a century. The GWP sediment stratigraphy is characterised by dark, organic-rich sediment with a defined basal clay layer, representing the local geology at 35 cm. Similarities between these cores show that the pond has not been disturbed and that the sediment archive is reliable. One core was then used as a master core for the rest of the analyses. Closely matched intra-basin χ_{LF} trends indicate minimal sediment disturbance and no periodic drying, suggesting a reliable depositional record, imperative for inferring a robust chronology (Fig. S2). An intact high-resolution sediment chronology for the 20th century was achieved using radiometric dating (Fig. S3, Tables S2 and S3). ^{210}Pb dates were determined for all sediment samples above the ^{210}Pb dating horizon, which in this core dated to 1926 (± 9 years) at a depth of 20.5 cm. The ^{137}Cs inventory indicates a well-resolved concentration peak at 12–13 cm (Fig. S3), which records the 1963 atmospheric fallout maximum from the testing of nuclear weapons. This is in good agreement with the ^{210}Pb results, which place 1963 (± 5 years) at a depth of 12.5 cm. The ^{210}Pb calculations also indicate that sedimentation rates were relatively constant from the mid-20th century through to the early 1990s (mean value 0.17 ± 0.03 g cm^{-2} $year^{-1}$) and have increased in recent years (mean post-1990 value of 0.25 ± 0.04 g cm^{-2} $year^{-1}$) (Table S2). Linear extrapolation of the ^{210}Pb chronology reveals an estimated basal date of ~1850 at 35 cm.

ICP-OES analysis was performed on 50 contiguous 1-cm samples down-core. The maximum, minimum and mean metal concentrations (mg g^{-1}) of Al, Ba, Cr, Co, Cu, Ga, Fe, Pb, Mg, Mn, Ni, K, Na, Sr, Ti and Zn are presented in Table 1. Al and Fe occurred at much higher concentrations than other metals as they are natural, geogenic components of the lake sediment matrix derived from the lake basin and catchment (Norton et al., 1992; Ma et al., 2016). Pb, Zn and Cu are typical proxies for anthropogenic pollution and exhibit different depositional trends to the minerogenic tracers (Al, Fe and Ti). The focal metal zinc was the 6th most abundant element measured, with average concentrations above those detected for Pb and Cu (Table 1). The highest concentration of zinc (0.486 mg g^{-1}, Table 1) occurred at 19 cm depth below which it steeply declined ($r = -0.679$, $p < 0.001$; Pearson's product-moment correlation coefficient) and remained elevated to the surface (≳0.3 mg g^{-1}) (Fig. 5).

Measures of total metal content can be potentially misleading in the context of biological studies as not all metal is bioavailable. We therefore employed the zinc specific biosensor *Pseudomonas putida* strain KT2440.2431(pDNPcadA1lux) (Hynninen et al., 2010) to

Table 1
Maximum, minimum and mean (± SD, n = 50) concentration (mg g^{-1}) of 17 heavy metals measured in the Griffin Wood Pond sediment core as quantified by ICP-OES.

Metal	Max	Min	Mean
Al	12.511	3.66	8.879 ± 2.09
Ba	0.213	0.056	0.123 ± 0.04
Co	0.013	0.002	0.007 ± 0.002
Cr	0.026	0.006	0.019 ± 0.004
Cu	0.699	0.007	0.169 ± 0.17
Fe	20.707	9.584	15.712 ± 2.45
Ga	0.181	0.067	0.123 ± 0.025
K	1.234	0.419	0.758 ± 0.197
Mn	0.65	0.119	0.297 ± 0.12
Mg	2.741	1.801	2.315 ± 0.18
Na	0.262	0.052	0.139 ± 0.04
Ni	0.044	0.011	0.027 ± 0.006
Pb	0.219	0.006	0.081 ± 0.06
Sr	0.034	0.002	0.01 ± 0.006
Ta	0.01	0.006	0.007 ± 0.0007
Ti	0.011	0.0001	0.005 ± 0.002
Zn	**0.486**	**0.02**	**0.178 ± 1.14**

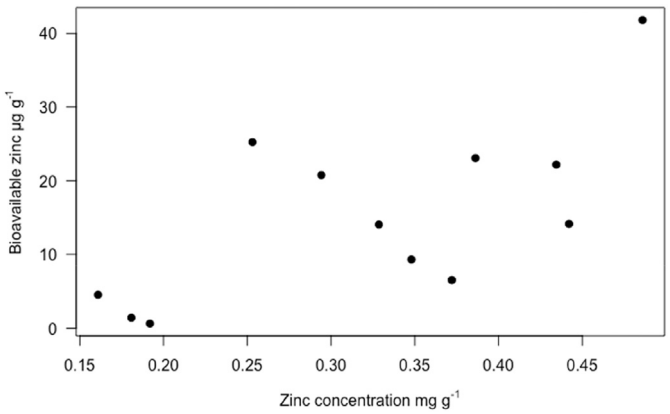

Fig. 2. Concentration of bioavailable zinc (mg g^{-1}) measured using the zinc specific biosensor *P. putida* strain T2440.2431 (pDNPcadA1lux) in twelve sediment samples. There is a significant positive correlation between total zinc measured by ICP-OES and bioavailable zinc concentration in the sediment ($r = 0.674$, $p < 0.05$).

determine the bioavailability of zinc down the core. Severe matrix effects due to the presence of toxicant mixtures that inhibit the biosensor gene expression resulted in the return of high-quality data for only twelve out of the fifty sediment samples. The highest concentration of bioavailable zinc (0.042 mg g^{-1}) was found at 20 cm depth, representing 8.6% of the total zinc concentration at that depth. The positive correlation between total zinc measured by ICP-OES and bioavailable zinc concentration in the sediment was statistically significant (Pearson product-moment correlation, $r = 0.674$, $p < 0.05$; Fig. 2) allowing the use of ICP-OES data as a proxy for relative differences in bacterial Zn exposure.

3.2. Pond sediment archive community diversity

Illumina 16S rRNA gene amplicons were obtained for sediment layers at 11–34 cm core depth (depths 31 cm and 35 cm were omitted due to insufficient reads) to characterise changes in bacterial community composition across a section of the sediment core representing a range of zinc concentrations. The evenness, richness and diversity of the sediment layer communities all significantly decreased with depth ($F_{1,22} = 6.2$, $p < 0.05$; $F_{1,22} = 88.7$, $p < 0.001$; $F_{1,22} = 8.2$, $p < 0.01$) (Fig. 3). However, the relative frequencies of the dominant phyla (*Proteobacteria*, *Chloroflexi*, *Bacteriodetes*, *Acidobacteria*, *Firmicutes* and *Aminicenantes*) were remarkably stable with depth (Fig. S6).

To quantify changes in the microbial community (β-diversity) across the core, pairwise Bray-Curtis community dissimilarities were contrasted with pairwise differences in depth and concentration of the 17 metals quantified by ICP-OES (Table 1). Microbial community dissimilarity between different layers of the core was explored using a principal coordinate analysis (PCoA) which showed that principal component 1 (PCo1) accounted for 49.96% of variation in community composition (Fig. 4). PCo1 correlated significantly with depth, but also correlated significantly with Ba, Co, Cr, Ni, Ta and Zn. PCo2 accounted for 20.94% of variation and correlated significantly with Cu, implying that this metal impacted community diversity differently than other metals. PCo3 accounted for 6.8% of variation and correlated highly significantly with Mn only (data not shown). A Mantel test was used to test for the effects of individual metals on ß-diversity (Mantel, 1967; Legendre et al., 2005). A significant positive linear correlation with ß-diversity was found for eleven metals, with zinc showing the strongest positive correlation with ß-diversity ($r = 0.800$, $p < 0.01$) (six metals did not show significant effects, Table 2 and Fig. S5). These data show there were consistent changes within microbial community composition over time through the core and suggest there could be several metals that had a significant effect on microbial community composition.

3.3. Zinc resistance and antibiotic resistance in culturable bacteria

To measure phenotypic zinc resistance of culturable bacteria, sediment from layers 1–50 cm was plated on nutrient agar containing the highest zinc concentration found in a test core extracted from GWP on the same day (0.625 mg ml^{-1}, Fig. S4) as well as on non-zinc amended agar. A moderate positive linear correlation was found between the proportion of zinc resistant bacteria and sediment zinc concentration (Pearson product moment correlation, $r = 0.416$, $p < 0.01$). As both changes in zinc concentration and changes in the proportion of zinc resistant bacteria were most pronounced around 11–35 cm depth (Fig. 5), a second experiment utilised clones isolated from these layers to test whether zinc resistance and antibiotic resistance were correlated. A total of 96 randomly selected clones per sediment layer were assayed for growth (resistance) or no growth (susceptibility) on agar amended with zinc (0.625 mg ml^{-1}), oxacillin (50 µg ml^{-1}), cefotaxime (50 µg ml^{-1}) and trimethoprim (60 µg ml^{-1}). Zinc concentration of the sediment layer of isolation was found to have a significant effect on proportion of zinc resistance in the randomly selected isolates (GLM, $F = 8.861$, $p < 0.001$) (Fig. 6a), consistent with the results of the first experiment. Linear regression analyses were performed to test the relationship between the proportion of zinc resistance and proportion of resistance to oxacillin (Fig. 6b), cefotaxime (Fig. 6c) and trimethoprim (Fig. 6d). To reduce the chances of type 1 errors, the extracted *p*-values ($p < 0.001$) from the linear regression analysis for all three antibiotics were bound together leaving a vector of *p*-values for Bonferroni correction. The proportion of zinc resistance among clones was highly significantly correlated to resistance against all three antibiotics ($p < 0.001$).

3.4. IntI1 prevalence and zinc resistance

The abundance of the class 1 integron-integrase gene, *intI1*, a genetic marker for anthropogenic pollutants (Gillings et al., 2015) was quantified using qPCR for sediment layers at 11–35 cm core depth. *IntI1* prevalence varied across depth (Fig. 7a), ranging from 0.12% at 25 cm to 3.66% at 20 cm. *IntI1* gene prevalence was found to correlate positively with zinc concentration as quantified by ICP-OES (Pearson product moment correlation, $r = 0.5579$, $p < 0.01$). There was a highly significant positive correlation between proportion of zinc resistant clones and *IntI1* prevalence (%) (Pearson's product-moment correlation $r = 0.779$, $p < 0.001$; Fig. 7b). A linear regression analysis was

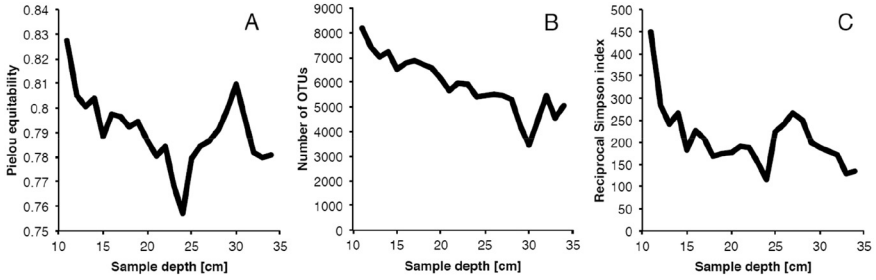

Fig. 3. Three measures of alpha diversity based on 16S sequencing for 23 sediment layers. A: Pielou equitability (measure of evenness), B: number of operational taxonomic units (OTU's) (measure of richness) and C: Reciprocal Simpsons Index (overall measure of alpha diversity).

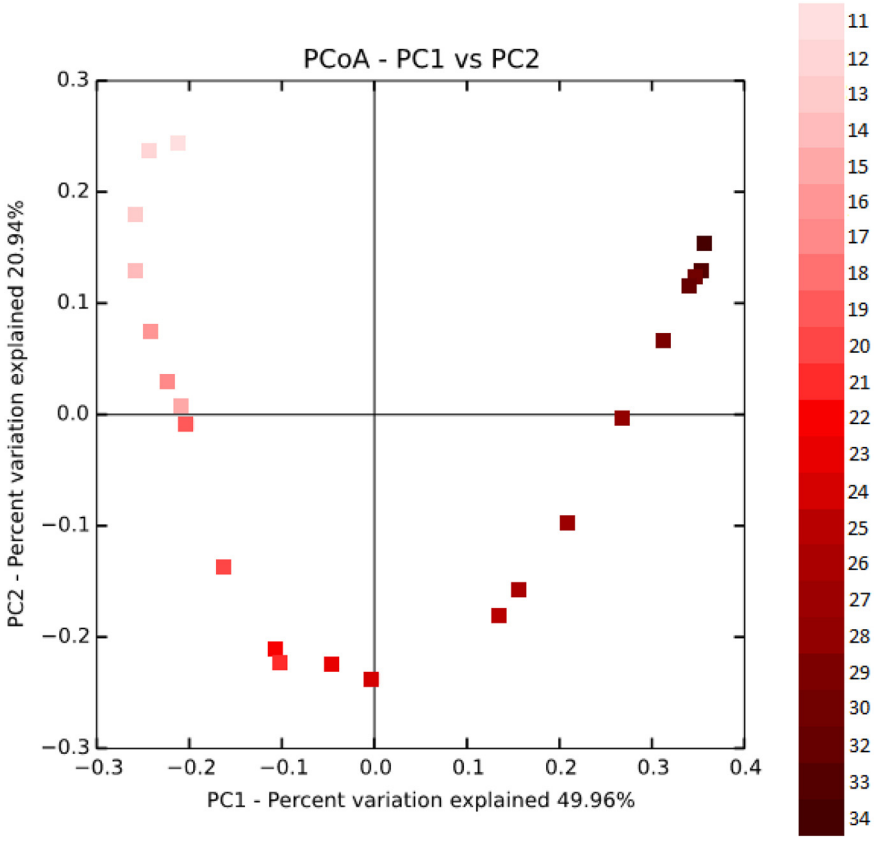

Fig. 4. Ordination plot showing grouping of sediment samples based on their microbial community structure. Each point represents a bacterial community from a layer of sediment with the shade of red darkening as depth increases. Principal component 1 (PC1) accounted for 49.96% of variation in community composition and principal component 2 (PC2) accounted for 20.94% of variation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Mantel test of the correlation between two distance matrices: beta diversity and the concentration of heavy metals present in the sediment core.

Metal	*r*	*p*-Value
Al	0.31458	0.001
Ba	0.54281	0.001
Co	0.43823	0.001
Cr	0.34444	0.001
Cu	0.29328	0.006
Fe	−0.04093	0.611
Ga	−0.08041	0.308
K	0.31765	0.002
Mg	0.25356	0.007
Mn	0.22372	0.01
Na	0.04114	0.619
Ni	0.34687	0.001
Pb	0.58627	0.001
Sr	0.27943	0.006
Ta	0.16119	0.051
Ti	0.11045	0.175
Zn	**0.80069**	**0.001**
Depth	**0.89686**	**0.001**

performed to test whether *intI1* gene prevalence was positively related with the proportion of zinc, oxacillin, cefotaxime and trimethoprim resistance in the 96 clones isolated from each layer (11–35). To reduce the chance of type 1 errors, extracted p-values from the linear regression analysis (oxacillin = $p < 0.001$, cefotaxime = $p < 0.001$, trimethoprim = $p < 0.001$) were bound together leaving a vector of *p*-values, after which a Bonferroni correction was performed on the extracted p-values. These data show that the proportion of zinc resistant isolates and cefotaxime resistant isolates correlated with *intI1* gene prevalence in the community ($p < 0.001$ in both cases). Resistance to oxacillin and trimethoprim was not significantly correlated with *intI1* gene prevalence ($p > 0.05$).

4. Discussion

Antibiotic resistance is a global health issue of utmost concern (Laxminarayan et al., 2013). Bacterial exposure to environmental pollutants such as heavy metals may co-select for antibiotic resistance, and could thereby significantly contribute to the emergence of antibiotic resistant bacteria (Alonso et al., 2001; Stepanauskas et al., 2005; Baker-

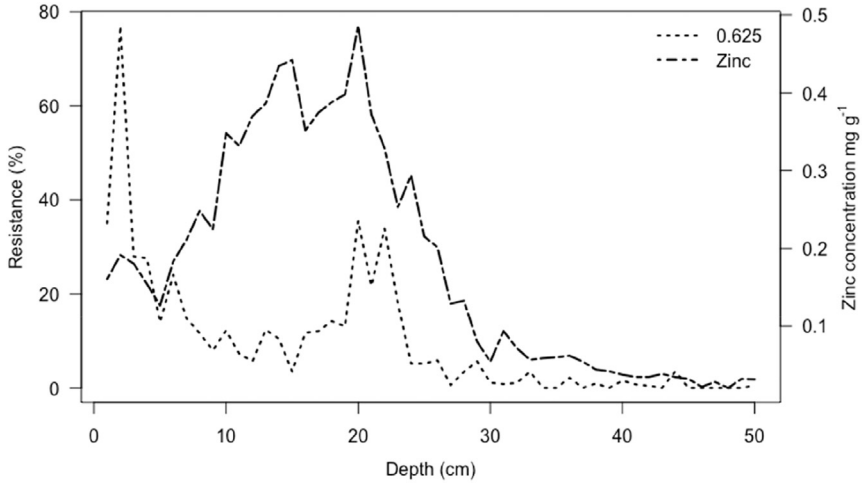

Fig. 5. Proportion of total culturable bacteria resistant to zinc (0.625 mg ml^{-1}) and zinc concentration (mg g^{-1}) found throughout the Griffin Wood Pond sediment core.

Austin et al., 2006; Garhwal et al., 2014; Gillings et al., 2015). Freshwater sediments are widespread, contain high bacterial densities and diversities and are frequently contaminated with heavy metals, meaning they likely form important reservoirs of antibiotic-resistant bacteria (Wright et al., 2006). We obtained a high-resolution record of pollution with 17 metals across a > 150 year-old sediment archive covering the rise of anthropogenic pollution to determine whether evolution of metal tolerance could be observed across these timescales, and whether this tolerance was related to metals concentrations within the sediment core and/or resistance to antibiotics.

Zinc, a common and toxic metal known to co-select for antibiotic resistance with industrial and agricultural activities (Calomiris et al., 1984; Peltier et al., 2010; Song et al., 2017) was chosen as a focal pollutant. Total zinc concentrations exhibited a period of elevation from 1926 to the early 1970s with a peak around 1939, likely reflecting the expansion and diversification of regional mining and industry. These anthropogenic activities are reflected by relatively high Zn concentrations when compared to other studies of highly polluted environments (e.g. Konstantinidis et al., 2003). Our results on bacterial community structure largely agree with a study on sediments from a Chinese river, where *Proteobacteria*, *Firmicutes*, *Bacteriodetes*, and *Acidobacteria* dominated both heavy metal-polluted and relatively unpolluted samples (Yin et al., 2015). The finding of *Aminicenantes* as a dominant Phylum fits with the prevalence of this taxon in sediments

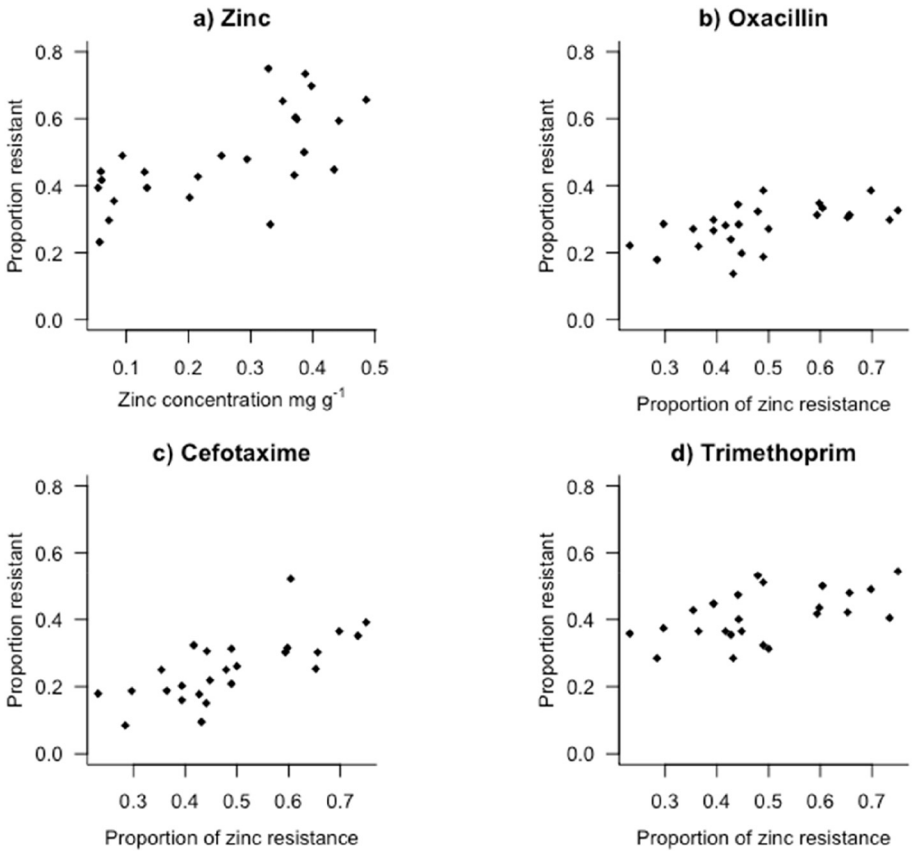

Fig. 6. Proportion of resistant bacteria to (a) zinc, (b) oxacillin (50 μg ml^{-1}), (c) cefotaxime (50 μg ml^{-1}) and (d) trimethoprim (60 μg ml^{-1}). Zinc concentration has a significant effect on proportion of zinc resistance (general linear model $p < 0.001$, m = 2.5894). Bonferroni correction on extracted *p*-values (b) $p < 0.001$ (c) $p < 0.001$ (d) $p < 0.001$ indicate a significant increase in tolerance to these antibiotics in isolates that exhibit resistance to zinc.

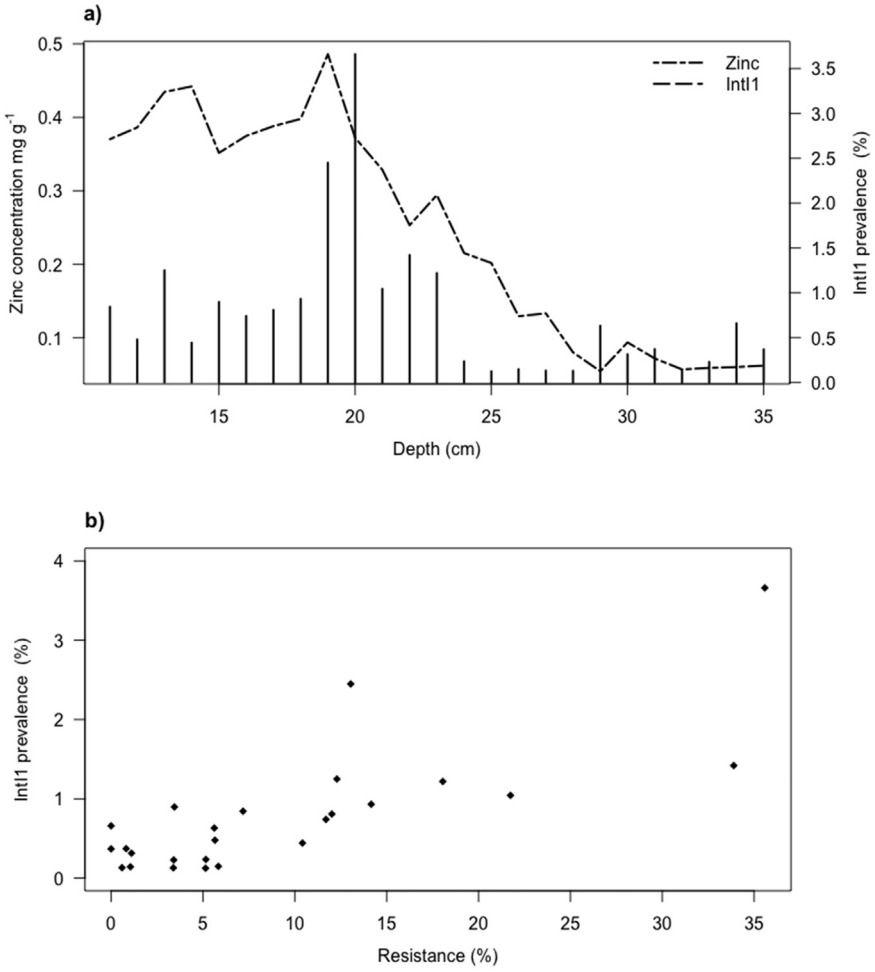

Fig. 7. a) Prevalence of the *intI1* gene (%) and zinc concentration (mg g^{-1}) as a function of core depth. b) Prevalence (%) of the *intI1* gene plotted as a function of proportion of zinc resistant clones (%) within samples 11–35. There is a significant strong positive linear correlation between resistance (%) and *IntI1* prevalence (%) ($R = 0.779$, $p < 0.001$).

polluted with metals and hydrocarbons (Farag et al., 2014). *Actinobacteria* have previously been reported to be an abundant component of metal-contaminated soils (Gremion et al., 2003) and the most prominent genus within *Actinobacteria* (*Streptomyces*) has been shown to be of high ecological importance, playing a major role in critical soil dynamics (Álvarez et al., 2013). Although we cannot exclude other abiotic factors such as pH, dissolved oxygen, or direct selection for antibiotic resistance via competitive interactions with antibiotic-producing community members, we were able to show that a variety of metals, most significantly zinc, play a significant role in structuring microbial community composition.

Bacterial zinc resistance mechanisms include reduced uptake, efficient efflux, internal or external sequestration and transformation to less toxic forms (Choudhury and Srivastava, 2001a, 2001b), which could result in cross-resistance to antimicrobials (Calomiris et al., 1984) (Bruins et al., 2000, Choudhury and Srivastava, 2001a, 2001b, Baker-Austin et al., 2006). For instance, a multidrug efflux transporter in *Listeria monocytogenes* was shown to reduce toxicity of zinc, cobalt and chromium and reduce the efficacy of cefotaxime (as well as clindamycin, erythromycin and josamycin) (Mata et al., 2000). Exposure to zinc at sub-toxic levels has been shown to result in increased resistance to the antibiotics tylosin, oxytetracycline and ciprofloxacin in bacteria in wastewater treatment reactors when those antibiotics are also present in the water (Peltier et al., 2010). Bacterial isolates from the sediment were used to test for patterns of cross resistance between zinc and cefotaxime, oxacillin and trimethoprim. The proportion of isolates exhibiting resistance to zinc was shown to significantly correlate with the proportion of isolates exhibiting resistance to all three antibiotics tested. The prevalence of the *intI1* gene across the core significantly correlated with the proportion of isolates with increased resistance to cefotaxime. However, oxacillin and trimethoprim resistance were not shown to have significant correlation with *intI1* gene prevalence. This may be a result of the mechanisms of resistance towards oxacillin and trimethoprim not being linked to the *intI1* gene.

One of the challenges facing the study of microbial communities is to unravel the complex mosaic of selective gradients that underlie observed changes in diversity (Koskella and Vos, 2015). Rather than taking a spatial approach comparing polluted and pristine sites, we took a temporal approach, comparing sediments of different ages and different pollution regimes to test for environmental metal-antibiotic co-selection. We demonstrate phenotypic resistance to clinically relevant antibiotics in bacteria isolated from sediment deposited before they were introduced (first-generation cephalosporins were only discovered in 1945 (Turck, 1982), oxacillin is a penicillin which only were used widespread from the 1960s (Fischer and Ganellin, 2010) and the first use of trimethoprim was in 1962 (Eliopoulos and Huovinen, 2001). As antibiotic resistance correlated positively with metal resistance and metal pollution, cross-resistance due to co-selection can be inferred as the most likely mechanism. It would be of great interest if our correlational approach could be followed up using microcosm experiments on sediments from the same location. For instance, fluorescently marked focal strains could be incubated in sediments collected from

different depths (with different metal concentrations) and re-isolated to quantify any changes in antibiotic resistance. Our data support the growing concern that heavy metal pollution functions as a selective agent in the maintenance of antibiotic resistance in the environment.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2019.105117.

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