Antibiotic selective pressure in microcosms: Pollution influences the persistence of multidrug resistant *Shigella flexneri* 2a YSH6000 strain in polluted river water samples

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ABSTRACT

Some urban rivers reach dangerous concentrations of residual antibiotics imposing a certain level of selective pressure on microorganisms to develop various antibiotic resistance mechanisms. In the current work, we have measured the persistence and growth of a multidrug resistant strain of *Shigella flexneri* 2a YSH6000 under a mock release of lethal concentration of oxytetracycline in microcosms of River Thames water. The water was sampled from upstream (lower levels of pollution) and downstream (higher levels of pollution) of London city centre. In our *in-vitro* microcosms, in the presence of 160 µg/mL of oxytetracycline, growth of *S. flexneri* in the downstream sector was up to 2 log(cfu/mL) higher relative to the upstream sector. This difference in growth is a sum of undefined interactions of different chemicals with the antibiotic. We extrapolated the contribution of two abundant pollutants in downstream sector: iron and phenanthrene. In the presence of selection pressure, iron at a concentration of 6.49 mg/L was found to foster the growth of resistant bacteria while phenanthrene at concentration of 160 µg/L reduced the growth of the resistant

strain. In addition, label free proteomics analysis showed that there are 64 proteins that were differentially expressed by the bacteria exposed to the upstream section versus the downstream sector. In the presence of oxytetracycline, at concentration of 160 µg/mL, the differences reduced to only a few proteins, demonstrating that environmental stress impacts protein synthesis. Such mock studies contribute to our knowledge of chemicals that reduce growth of resistant strains and aids in the identification of selective biomarkers.

Keywords: anthropogenic pollution, river, iron, polycyclic aromatic hydrocarbons, phenanthrene, *Shigella flexneri*, tetracycline

1. INTRODUCTION

It is well known that antibiotic selective pressure in the environment may lead to the development of dangerous resistant bacterial strains (EMA, 2018; Ashbolt *et al.*, 2013). The indiscriminate use of antibiotics, both in clinical and agricultural milieu has brought a rapid dissemination of antibiotic resistance genes (ARGs) in rivers (Hong *et al.*, 2018; Di Cesare *et al.*, 2016). Numerous rivers around the world are contaminated with hazardous levels of antibiotics (Brack and Schulze, 2019). Antibiotics released into the environment through water closet (WC) flush, animal waste and sewage dispersal, find their way into rivers and soil (Hanamoto *et al.*, 2018). Waste released directly into rivers is a significant problem. This is a serious issue in developing countries, as well as in modern cities. Wastewater treatment plants (WWTPs) are sources of ARGs and antibiotics into the environment (Brown *et al.*, 2019). Even

when appropriate WWTPs are in place, during heavy rainfall, combined sewer overflows (CSOs) discharges a mixture of sewage and run-off into rivers which may be polluting in nature (Group, 2005).

A recent study on hotspots of antibiotic resistance selection in rivers from 517 urban locations in 61 countries has shown that the most frequently detected compound was ciprofloxacin followed by trimethoprim and sulfamethoxazole (Boxall, 2019). Currently only rivers in 31 countries have been studied. Of these, the concentrations of antibiotics in 19 of them were below risk of Antimicrobial Resistance (AMR) selection. Rivers in Austria, Belgium, Cyprus, Iraq, Laos, Malaysia, Nigeria, Pakistan, Portugal, UK, USA had mean concentrations of at least one antibiotic above the 'predicted no effect concentrations'(PNEC) for antimicrobial resistance indicating that resistance selection is possible (Boxall, 2019).

Current methods that assess the risk of AMR occurrence include spatial and temporal distribution of chemicals in the environment, quantitative Polymerase Chain Reaction (qPCR) and growth-based methods indicating PNECs (Murray, Stanton and Zhang, 2019). However, to support the development of risk assessments and PNECs indicators, it is also necessary to study mock occurrences of lethal release of antibiotics into rivers and the behaviour of resistant bacteria. In this work we have studied the growth of a multi-antibiotic resistant *Shigella flexneri* strain at lethal concentration of oxytetracycline in River Thames water to demonstrate what may happen if this level of antibiotic was found to occur in the river.

For this purpose, we used microcosms defined as simplified ecosystems to study the behaviour of a natural niche. For obvious reasons we could not release such dangerous strains into the real environment and study their persistence, therefore an *in-vitro* approach was followed. It is very important to understand the persistence and fate of a multidrug resistant strain in urban rivers, in particular due to the dangerous rise of antibiotic resistances in hospitals and highly populated urban areas. It is highly unlikely that these dangerous strains will be found in rivers, but in the unlikely event that they were, what impact could this potentially have? To the best of our knowledge, there are no studies that have reported this.

In this study, the tetracycline resistant strain *Shigella flexneri* 2a YSH6000, isolated from a patient, was used as a model bacterium to examine the effect of the chemical environmental pollutants in river water. *S. flexneri* was chosen because of its very low infection dose (tens of cells) (Baveja, 2014; Sasakawa *et al.*, 1986). Oxytetracycline was used to induce selective pressure. Microcosms from River Thames (London, UK) were used as an environmental model of a highly populated metropolis in Europe. Our experiments were designed to identify the persistence and growth of *S. flexneri* in this complex environment in the presence and absence of oxytetracycline as a selective pressure, and to extrapolate the effect of single pollutants on the replication of *Shigella* in presence of the antibiotic.

Here we show in the microcosms that complex chemical pollution footprint (Zijp, Posthuma and van de Meent, 2014) in a river plays a key role in the bioavailability of oxytetracycline. The mixture of chemical pollutants plays different roles in the overall growth of bacteria in the presence of a selective pressure. Comparative metaproteomic analysis reveal lethal concentrations of oxytetracycline repress several proteins involved in different metabolic cascades.

2. MATERIALS AND METHODS

2.1 Sampling sites. River water was sampled at two locations of the River Thames (London, UK): upstream and downstream of the city centre (geospatial coordinates are available (Maruzani *et al.*, 2018)). Sampling sector were approximately 40 km apart . For each sampling point, three 2 L samples were collected in different parts of the river. The sampling was performed by sampling water in polyethylene terephthalate bottles. The samples were frozen within 7 h of sampling.

2.2 Strain used in this study. The strain used in this study is *Shigella flexneri* 2a YSH6000 (Rajakumar, Sasakawa and Adler, 1996). It harbours a 66 Kbps resistance locus pathogenicity island (SRL PAI) containing a 16 Kbps SRL region which encodes for resistance to streptomycin (*aadA1*), ampicillin (*oxa-1*), chloramphenicol (*cat*) and tetracycline (*tetRA* - efflux pump and receptor) (Sasakawa *et al.*, 1986). The bacterium was cultured overnight in Lysogeny Broth (LB) medium (Oxoid, Basingstoke, UK), or in 1 X Minimal Salt (M9 medium) (Invitrogen, Carlsbad, US). M9 medium was prepared according to manufacturer's specifications and enriched with 12.5 µM nicotinic acid (Sigma-Aldrich, Dorset, UK) (*S. flexneri* 2a YSH6000 is auxotroph for nicotinic acid) and 0.2 % w/v of glucose (Sigma-Aldrich, Dorset, UK) to produce the final M9 medium. Glucose (0.2 %, w/v) was added to strains that were grown in filtered River Thames water in order to support growth.

2.3 Filtration of the River Thames water samples. Aliquots (200 mL) of water from each sampling site were filtered twice using Whatman paper No 1 (particle retention 11 µm) (Sigma-Aldrich, Dorset, UK) then twice using 0.22 µm filters (Billerica, MA, USA). Subsequently, the three samples from the same river sector were combined to obtain "upstream" and "downstream" samples. These were subsequently aliquoted into 50 mL Falcon tubes (Fisher, Basingstoke, UK) and frozen at -20 ºC until analysis.

2.4 Degradation of tetracycline. M9 overnight culture of *S. flexneri* was washed three times with fresh M9 medium or River Thames water before being used as inoculum. A small volume (30 µL) of 10^8 cell/mL (the initial inoculum was estimated via serial dilutions and CFU counting on LB plate) was inoculated into 2 mL of M9 medium or River Thames (enriched with 0.2 % w/v of glucose) with 1.5 µg/L of oxytetracycline (Sigma-Aldrich, Dorset, UK). The microcosms were incubated at 30 ºC for up to 72 h without shaking. The microcosms were then filtered using 0.22 µm filter (Billerica, MA, USA). The concentration of oxytetracycline in the filtrate was measured using high performance liquid chromatography with tandem mass spectrometry. The instrumentation and conditions that were used for measurement of

oxytetracycline were identical to those reported previously (Maruzani *et al.*, 2018). Briefly, an X-LC UHPLC system (Jasco, Great Dunmow, UK) coupled to an API 3000 triple quadrupole mass spectrometer (Applied Biosystems, Warrington, UK) was used. The software used to acquire data and set the instrument parameters was Analyst version 1.4.2 (Applied Biosystems, Warrington, UK). The chromatographic separation was achieved using a Onyxmonolithic C18 100 x 3.0 mm i.d. connected to a 5 x 3.0 mm guard cartridge containing the same stationary phase (Phenomonex, Macclesfield, UK). A linear binary gradient composed of A - 0.1 % formic acid in water and B - 0.1 % formic acid in acetonitrile was used. The elution profile used started at 10 % B then increased to 75 % over 5 min and was held at this level for 1 min. The flow rate was set at 0.21 mL/min. An injection volume of 10 µLwas used and the column oven temperature was maintained at 50˚C. The MS was equipped with an electrospray ionisation source which was operated in the positive-ion mode. The MRM transitions for oxytetracycline of *m/z* 445.3 → 410.1 and 445.3 → 154.5 were monitored simultaneously. The detection limit (LOD) was established as the lowest concentration of the calibration standard that was detected with a signal-to-noise (S/N) ratio ≥ 3:1 while the quantification limit (LOQ) was established as the lowest concentration of the calibration standard that was detected with a signal-to-noise (S/N) ratio ≥ 10:1. The LOD and LOQ were 2 and 10 ng/mL respectively. The retention time of the antibiotic was found to be 3.1 min. Oxytetracycline was identified by retention times (Rt) and by two selected reaction monitoring (SRM) transitions.

2.5 Heavy metal analysis of River Water. Quantitation was performed using an ICP-MS (X series II, Thermo Scientific, UK) using a 20 µg L^{-1} indium as an internal standard. The instrument was tuned daily using a 10 µg/L^{-} mixture of indium, cerium, cobalt, uranium and lithium solution. All dilutions and external calibration standards were prepared using deionised water with a conductivity of 18.2 MΩ obtained from a Purite water purification system (Thame, UK). Magnesium, potassium, lithium, manganese, iron, copper, zinc, indium and cadmium solutions and nitric acid (trace metal analysis) were obtained from Fisher Scientific

(Loughborough, UK). Method quantification limits (MQL) were calculated by multiplying the standard deviation signal by 10 (at the retention time of the quantification species) of seven consecutive blank samples. Method detection limits (MDL) were calculated by multiplying the standard deviation by 3.14 times the signal of seven consecutive blank samples to achieve 99 % confidence intervals (CI). PlasmaLab software (ThermoFisher Scientific, Hemel Hempsted, UK) was used to sen instrument parameters and analyse the data.

2.6 Proliferation of Shigella flexneri in River Thames microcosms. Aliquots (200 µL) of filtered water from upstream and downstream (enriched with 0.2% w/v glucose) was added to a sterile 96-well microplate. Each well was inoculated with approximately 200-500 cells of *S. flexneri* (the initial inoculum was estimated via serial dilutions and CFU counting on LB plate). The plate was incubated at 30 ºC for 48 h. After incubation, 25 µL of sample from each well was spread onto LB plate or Hektoen Enteric Agar (to confirm absence of contamination) and log(CFU/mL) was calculated for the CFU obtained from LB agar plates. *S. flexneri* proliferation was also measured in River Thames non diluted (ND), as control, and diluted 1:2 and 1:20 using distilled water enriched with 0.2% (v/v) sterile glucose to support growth. CFUs were obtained as described above.

For the experiments with iron, *S. flexneri* was again cultured in the presence of $FeCl_3$ (Fisher Scientific, Leicestershire, UK), the iron was added to the wells at a final concentration of 6.48 mg/L. The experiments were carried out in presence of 160µg/mL oxytetracycline. Growth was measured by counting CFUs as described above. Experiments were carried out with at least three biological and two technical replicates.

2.7 Growth with oxytetracycline and PAHs. Small volumes (200 µL) of M9 medium was added to sterile 96-well microplate and enriched with 0.2% (w/v) sterile glucose and 12.5 µM nicotinic acid to support growth. When appropriate, 160 µg/mL of oxytetracycline and 140 µg/L each of phenantherene, dibenz(a,h)anthracene, pyrene, anthracene, benzo(a)pyrene (Sigma-Aldrich Dorset, UK) were added. Each well was initially inoculated with approximately 200-500 cells

of *S. flexneri*. The plates were incubated in a microplate reader GMB LabTech set at 30 ºC with absorbance measured using OD=600 nm at 0, 7 and 13.5 h. For experiments that were repeated with upstream and downstream River Thames samples, water was enriched with 140.1 µg/L phenanthrene. Control wells with no phenanthrene were included here. It has to be noted that in upstream water the basal level of phenanthrene was 1.1 µg/L (Maruzani *et al.*, 2018). The study included three biological and two technical replicates.

2.8 qPCR to verify the expression of tetAR genes in S. flexneri. One (1) mL from overnight cultures of *S. flexneri* in M9 medium was centrifuged and pellet was washed three times and the pellet was re-suspended in 4 mL of upstream, downstream or M9 medium. All microcosms were enriched with glucose and nicotinic acid. Oxytetracycline (160 µg/mL) was added when appropriate. Controls did not contain oxytetracycline. Microcosms were incubated at 30 ºC without shaking for 48 h. Total RNA was extracted from samples using mirVana™ miRNA Isolation Kit (Life Technologies, Carlsbad, CA, USA) using the manufacturers' instructions. RNA integrity was visualized on 1.3 % agarose gel electrophoresis. Samples were quantified using a Nanodrop spectrophotometer (ThermoFisher Scientific, Loughborough, UK) in accordance with the manufacturers' instructions. DNA was degraded using TURBO DNA-freeTM Kit (Life Technologies). cDNA synthesis was performed using a qPCRBIO cDNA Synthesis Kit (PCRBio) in accordance with the user manual guide using random hexamer primers. qPCR was performed on a qPCR LightCycler 96 System (Life Technologies, Carlsbad, CA, USA) by using PCRBIO SyGreen Mix Hi-ROX (PCR Biosystems, London, UK). A negative control was obtained by using PCR grade water in place of a cDNA template. DNA-free RNA was tested via standard PCR amplification to ensure complete removal of genomic DNA prior to cDNA generation by using 16S primers (Marvasi *et al.*, 2009). *Shigella acnB* gene was used as an internal reference gene. qPCR was performed by using the following cycles: initial denaturation at 95°C for 2 min, 40 cycles of denaturation at 95°C, annealing at 60°C and extending at 65°C for 30 s. Primers used in PCR reactions are shown in Supplementary Material S2. Minimum requirement tests were carried out to ensure specific

amplifications were performed as recommended by the MIQE Guideline (Bustin *et al.*, 2009). PCR amplification efficiency was established by means of calibration curves. Livak ($2^{-\Delta\Delta Ct}$) method was used to analyse genes expression. Experiments were conducted with at least three biological and two technical replicates.

2.9 Comparative proteome analysis. An overnight *S. flexneri* strain grown in M9 medium was diluted until OD_{600} was 0.20 absorbance units. A small volume (1 mL) was inoculated into 6 mL of microcosms containing upstream, downstream water and M9 enriched with 0.2% w/v glucose. In parallel, the same microcosms were treated with 160 µg/mL of oxytetracycline. The microcosms were incubated for 8 h at 30 ºC in a static incubator. Tubes were centrifuged and cells were re-suspended in sterile 50 mM EDTA. The cells were sonicated at amplitude 7.2 with 6 cycles of 30 s each, with 1 min in ice between the cycles. Cellular fragmentation was verified using a microscope. Cell debris was removed by centrifuging samples at 13,000 *x g* for 10 min. The supernatant was mixed with 4 volumes of cold acetone and then left for 1 h at -20 ºC. After incubation, the samples were centrifuged at 13,000 *x* for 10 min. Supernatant was discarded, and the pellet was dried and re-suspended in 50 mM EDTA. Proteins were then reduced, alkylated and subsequently digested using 2 µL of trypsin (0.1 µg/µL dissolved in 50 mM ammonium bicarbonate) at 37 °C for 3 h. A further 2 µL of trypsin was added to the samples and the mixture was incubated at 37 °C for an additional 2 h. The samples were diluted in 150 µL 50 mM ammonium bicarbonate and passed through Mini-Uni Prep filter devices.

Mass Spectrometry. Tryptic digests were analysed using a Dionex Ultimate 3000 RSLC Nano ultra-high performance liquid chromatography system coupled to a Q Exactive mass spectrometer. Aliquot (15 µL) of tryptic digest was desalted and concentrated using a 5 mm x 300 µm i.d. C18 trap cartridge and solvents composed of a mixture of water and acetonitrile (98:2 %, v/v) containing 0.05 % TFA (loading solvent A) and a mixture of acetonitrile and water (80:20 %, v/v) (loading solvent B) at a flow rate of 20 µL/min. The concentrated sample was separated using a binary gradient elution profile composed of a mixture of water and

acetonitrile (95:5,v/v) containing 0.1 % formic acid (eluent A) and a mixture of acetonitrile and water (80:20 %, v/v) (eluent B) containing 0.1 % formic acid at a flow rate of 6 µL/min. The gradient was 0 min- 0 %B, 4 min- 5 %B, 5 min- 8 %B, 40 min-40 %B, 41 min-80 %B. The autosampler and column oven temperature was set to 4 and 40 °C respectively. The Q Exactive was operated in a data dependent mode. MS survey scans were acquired from *m/z* 350 to 2000 at resolution of 70,000 with AGC of 3e6 and maximum IT of 100 ms. The 20 most abundant ions were subjected to MS/MS and measured with a resolution of 17,000 and AGC of 1e5 and maximum IT of 50 ms.

LC-MS/MS data was processed using PEAKS Studio software v8.5 (Zhang et al., 2012) with database searching against a downloaded FASTA file originating from Uniprot_SwissProt_2019_02. A protein identification score -10lgP of >20 was used as the cut-off for confident identification. Quantitation was then undertaken using the PEAKSQ algorithm and several two group comparisons were performed using pairwise comparisons of all samples available within the experimental groups. Heatmaps displaying quantitative changes were used to visualize changes in protein abundance.

2.10 Statistical analysis. T-tests, Anova or Tukey's mean separation tests were performed by using JMP statistical software (SAS Institute, Cary, NC, USA). Graphs were drawn using Prism 8.0.

3. RESULTS

S. flexneri growth in river water microcosms is affected by pollution.

The sampling was performed at two sections of River Thames, about 20 km *upstream* and *downstream* from the London city centre. Mock occurrence was simulated in microcosms with upstream and downstream river water enriched with 0.2% w/v of glucose to speed up *S.*

flexneri growth. We tested to what extent pollution was able to affect the growth of *S. flexneri* in the presence of lethal concentrations of oxytetracycline. The pollutants present in the river water were partially characterized and found to contain benzo(a)pyrene, pyrene and phenanthrene at 128, 171 and 128 times higher in downstream sector as compared to upstream sector (our previous work (Maruzani *et al.*, 2018)). Also, the concentration of iron was found to be 105 % higher in the downstream section compared to the upstream section (Table 1).

Initially, we measured the growth of *S. flexneri* in the presence of oxytetracycline at a range of concentrations from 10 to 360 µg/mL (Figure 1A). The measurement showed that in the presence of up to 160 µg/mL of oxytetracycline *S. flexneri* grew up to 2 log(cfu/mL) more in downstream compared to upstream water microcosms (Figure 1A). Downstream River Thames water was diluted 20 fold showing that reduction of growth of *S. flexneri* was dependent on the concentration of chemicals in the environment (Figure 1B). We interpreted this result as differences in bioavailability of oxytetracycline, hypothesising that the pollutant mixture interacts with oxytetracycline removing its toxic effect.

To assess bioavailability of the antibiotic, *tetAR* genes harboured in the 16 Kbps SRL region of *S. flexneri* were used as bioindicator for oxytetracycline, where expression of the genes would indicate bioavailability of the antibiotic. Interestingly, *tetRA genes* were not differentially expressed in either upstream or downstream samples with reference to the internal gene control, while showing higher expression in the minimal M9 medium (Table 2). We therefore confirmed that bioavailability of oxytetracycline is strongly reduced in River Thames microcosms but not in M9 medium.

Anthropogenic phenanthrene at 140 µg/L reduced the growth of S. flexneri.

Downstream river water contains a complex mixture of thousands of molecules, we focused our attention on six chemicals: phenanthrene, pyrene, anthracene, benzo(a)pyrene and benzo(a)anthracene (and iron (Table 1)). To study the effect of each compound in association with oxytetracycline on the growth of *S. flexneri*, microcosms were generated in M9 medium.

304 *S. flexneri* growth in M9 medium enriched with and without 160 µg/L of oxytetracycline did not
305 show any significant difference in proliferation (Figure 2, A). However, in the presence of both
306 140 µg/mL phenanthrene and 160 µg/mL oxytetracycline the growth of the organism was
307 significantly delayed when compared with the control (Figure 2, B). The other PAHs with or
308 without oxytetracycline did not affect growth of *S. flexneri* (Figure 3).
309 We enriched the upstream River Thames water microcosms with the same concentration of
310 phenanthrene (140 µg/mL) as that found in downstream River Thames water, as in (Maruzani
311 *et al.*, 2018)). Growth of *S. flexneri* was measured, but no significant differences were found
312 (Figure 4 A). Interestingly, when upstream water was enriched with both oxytetracycline and
313 phenanthrene the growth of *S. flexneri* was significantly reduced (Figure 4 B), as previously
314 observed in M9 medium (Figure 2 B).
315
316 *Anthropogenic iron at 6.49 mg/L supports growth of multidrug resistant Shigella flexneri.*
317 As previously described, heavy metals were measured in upstream and downstream river
318 water (Table 1). The level of iron was 105 % more concentrated downstream when compared
319 to upstream. The higher concentration of iron in downstream water could be involved in
320 supporting the growth of *S. flexneri* at selective pressure of oxytetracycline at concentration
321 of 160 µg/mL observed in Figure 1. Therefore, growth of *S. flexneri* in upstream microcosm
322 enriched with iron (III) chloride ($FeCl_3$) was tested (Figure 5). The experiment showed that
323 increasing the concentration of iron in the upstream microcosms to the same concentration as
324 in the downstream microcosm significantly supported the growth of *S. flexneri* to about 0.1
325 log(cfu/mL) (Figure 5). *S. flexneri* grown in the absence of tetracycline did not show any
326 significant difference in growth when the same concentrations of $FeCl_3$ was added. No
327 degradation of oxytetracycline was observed in the presence of 6 and 12 mg/L of $FeCl_3$ over
328 3 days (Table 3).
329
330

Differential proteomics analysis of S. flexneri in upstream versus downstream River Thames suggests a response to stress conditions

Microcosms were also used for differential proteomics analysis of *S. flexneri* in upstream versus downstream River Thames water in the presence and absence of oxytetracycline. The same differential analysis was repeated using M9 medium to measure changes in a minimal medium. A total of 555, 291 and 552 proteins (98564, 3703 and 9074 peptides) were detected from M9, downstream and upstream, respectively. Among all samples and replicas 513 proteins were consistently detected in all replicates and treatments. Unique proteins were highlighted in global intensity maps of fold changes (Figures 6 and 7).

The proteome of *S. flexneri* grown in upstream sector in presence and absence of oxytetracycline showed 12 proteins differentially expressed. Among them 11 proteins were repressed in presence of oxytetracycline (Figure 6 A). Proteins of interest were: a histidine-binding periplasmic protein [P0AEU1], phosphopentomutase [B7LNS3], a signal recognition particle protein targeting and insertion of membrane proteins into the cytoplasmic membrane [P0AGD7], a peroxide stress resistance protein [B1XBD1], two outer membrane proteins [P24016, P0A906], a protein involved in cell division [P0A9A8], oxidoreductase [P37440], in addition to ribosomal proteins [Q328J9]. The 50S ribosomal protein L2 was upregulated in presence of oxytetracycline [B1X6G8].

When *S. flexneri* cells were exposed to downstream water with and without oxytetracycline, only three proteins were significantly differentially regulated in presence of tetracycline (Figure 6 B): a 30S large ribosomal subunit [B1IPY8], a S-ribosyl homocysteine lyase [B0TR04] of *Shewanella halifaxensis* (a gamma-proteobacteria), used to communicate both the cell density and the metabolic potential of the environment. The third proteins is a cold shock-like protein CspC [P0A9Y7].

The comparison between upstream and downstream River Thames without addition of oxytetracycline showed the highest variability (64 proteins) (Figure 7 A). In upstream microcosms only three proteins were significantly repressed: Two of them are proteins

involved in the construction of the 50S large ribosomal subunit [B4TKL0, B1X6G8]. The third protein is involved in the fatty acid biosynthesis pathway, which is part of the lipid metabolism [P0ABD8]. Sixty-one proteins related to stress were upregulated in upstream samples such as: superoxide dismutase [P66828], hydrolases [P21367], ATP-dependent zinc metalloprotease involved in the quality control of integral membrane proteins [P0AAI4] and in general factors for protein synthesis, including serine-tRNA ligase for the attachment of serine to tRNA [Q0T8M9]. The elongation factor G was also overexpressed upon exposure in upstream water [Q0SZX7]. Proteins downregulated in upstream sector were involved in the construction of the 50S ribosome and a biotin carboxyl carrier protein of acetyl-CoA carboxylase [P0ABD8] (Figure 7 A). Once oxytetracycline was added, five proteins were significantly differentially expressed (Figure 7 B). Proteins of interest were: the outer porin protein X (*ompX*) significantly more expressed in downstream water [P0A920]. In addition a NAD(P)H dehydrogenase and a component of the ribosome were more expressed in upstream [respectively, P0A8G7 and C0PZP0]. Comparison performed in M9 with and without oxytetracycline showed repression of two proteins involved in the ribosomal 50S [B4TKL0, B7MCT2].

4. DISCUSSION

Environmental risk assessment in terms of selection for antimicrobial resistance is not performed because there are no ecotoxicological assays with selective endpoints to determine the concentrations of antibiotics that select for AMR (Murray, Stanton and Zhang, 2019). Therefore, the study of mock occurrences of lethal concentrations in river water (or other environments) can provide useful information about the fate of a strain or antibiotics in a specific environmental context. There are at least three important reasons for carrying out this study and these were to: i) assess the chemical fingerprinting of pollution on growth of

386 resistant strains; ii) determine whether a single chemical can trigger the growth of resistant
387 microbes under selective pressure; and iii) better visualize the physiology of the resistant
388 strains through transcriptomics, proteomics and metabolomics analysis to identify biomarkers.
389 In this work, we have only started to explore all these dimensions. We approached the study
390 by using a closed ecological system, a microcosm. Whilst we are aware that such simplified
391 solution may not completely reflect an open system, such as a river, the result obtained in this
392 study would pose a foundation for future analysis and confirmation in the real environment. It
393 would also be impossible to study the release of human pathogens in the environment for
394 similar purpose. Our findings also highlight how the selected chemicals can play a pivotal role
395 in a real environment that could vary in magnitude. The assessment of the chemical
396 fingerprinting on persistence of multidrug resistant bacteria is extremely important. The
397 downstream pollution mixture was found to supports growth increase up to 2 logs (cfu/mL)
398 compared with the upstream water sector in presence of 160 µg/mL of oxytetracycline. Since
399 the antibiotic is not degraded in downstream water, as shown in Table 3 and in our previous
400 study (Maruzani *et al.*, 2018), there may be other factors such as complexation of the antibiotic
401 to other chemicals/constituents which prevents tetracycline from inhibiting the growth of *S.*
402 *flexneri*. The genes *tetRA* can be used as biomarkers of tetracycline (Moller *et al.*, 2016).
403 Therefore, reduced bioavailability of oxytetracycline was confirmed by measuring the
404 expression of *tetAR* genes which were not expressed in presence of the antibiotic in the river
405 water but were detected in the M9 medium.
406 In an effort to determine which molecules affect the availability of oxytetracycline in the
407 downstream water microcosms we performed chemical quantification of a pool of a number
408 of pollutants ((Maruzani *et al.*, 2018) and Table 1). Two candidates attracted our attention,
409 phenanthrene and iron. The concentrations of both were found to be higher in downstream
410 compared to upstream sector. Both chemicals were tested to see to what extent they support
411 the growth of *S. flexneri* in the presence of oxytetracycline. The experiments showed the two
412 had opposite effects. In the presence of lethal selective pressure, phenanthrene reduced the
413 growth (Figure 4) while iron, promoted it (Figure 5). With reference to phenanthrene, the efflux

pump could be involved in the detoxification of phenanthrene in competition with tetracycline, reducing therefore the ability of the pumps to detoxify the antibiotic – and thereby reduced growth. There are examples in literature of pumps that extrude PAHs: EmhABC efflux pump efficiently extruded phenanthrene in *Pseudomonas fluorescens* strain LP6a (Adebusuyi and Foght, 2011; Adebusuyi *et al.*, 2012). Recently, PAHs have also been found to support selection of antibiotic resistance. Presence of 100 mg/L of naphthalene or 10 mg/L of phenanthrene significantly enhanced the abundance of class I integrase gene (*intI1*), sulfanilamide resistance gene (*sulI*), and aminoglycosides resistance gene (*aadA2*) in the microbial community of coastal water (Wang *et al.*, 2017).

On the other hand, iron was found to promote growth. It has been shown that Fe(III) ions in aqueous solution in the absence of light induced degradation of three tetracycline antibiotics, tetracycline, oxytetracycline and chloro-oxytetracycline (Wang *et al.*, 2015). Also, Wang et al [21] demonstrated that Fe(II) complexes with tetracycline, oxytetracycline, or chloro-oxytetracycline which could simultaneously lead to the accelerated oxidation of Fe(II) to Fe(III) and faster degradation of tetracyclines (Wang *et al.*, 2016). Although, the Fe(III) cannot be oxidised further, in some cases it can be reduced to Fe(II) again by tetracyclines (Wang *et al.*, 2016), supporting the role of chemicals in the environment that ultimately leads to the equilibrium of Fe(II)/Fe(III). However, in our experimental setting, oxytetracycline was not degraded in the presence of iron Fe(III) within 3 days at 30ºC (Table 3), showing the importance of the chemical environment in supporting or preventing these redox reactions. Because River Thames is a complex environment, we also found that other organic molecules can compete and chelate with iron, for example humic acids. These are present in River Thames and can compete with tetracyclines for Fe(II) (Wang *et al.*, 2016; Watt *et al.*, 1996; Old *et al.*, 2019) in the open system. Ultimately, the current study has shown that excess of iron may foster growth of resistant bacteria even in presence of selective pressure.

Only a few studies have focused on studying tetracycline related stress response on the proteome of different strains such as *E. coli and Coxiella burnetii* (Vranakis *et al.*, 2012; Xu

et al., 2006; Jones-Dias *et al.*, 2017) and to our knowledge this is the first report for *S. flexneri*. Jones-Dias and collaborators studied the response of soluble proteome of the environmental-borne *Escherichia coli* EcAmb278 to tetracycline. These authors found that treatment with tetracycline resulted in 12 proteins being differentially regulated, showing more than two-fold change (Jones-Dias *et al.*, 2017). In the current study, we also found 12 proteins in *S. flexneri* that were differentially regulated when the organism was cultured in upstream water enriched with oxytetracycline (Figure 7 A). However, the proteins that were identified were different to those reported by Jones-Dias. This can be attributed to different culture conditions and different strains (River Thames water versus MacConkey agar) (Jones-Dias *et al.*, 2017; Sims and Kim, 2011).

In the absence of any selective pressure a comparison of organism cultured in upstream versus downstream microcosms showed 64 proteins that are differentially regulated. In our experiment, exposure of *S. flexneri* to oxytetracycline appeared to repress protein transcription which suggests a response to stress conditions and the toxic effect of oxytetracycline. Some proteins have no benefits in an environment incurring a quantifiable fitness costs on cellular growth rates. Certainly, the magnitude and variability of unused protein expression is largely due to the challenge in determining environment-specific proteome utilisation, and unused protein abundances are not constant across environments (O'Brien, Utrilla and Palsson, 2016). In our study, the addition of oxytetracycline to any tested environment (upstream, downstream and M9 medium) reduced the number of differently regulated proteins. Reduction of proteins accounts for slow replication and it is known that slower growing cells are more resistant to cellular stresses, such as response to antibiotics (Poole, 2012; O'Brien, Utrilla and Palsson, 2016).

Conclusions

A number of authors have provided guidelines for risk assessment related to the development of AMR in environment (EMA, 2018; Ashbolt *et al.*, 2013). Antibiotic resistant bacteria (ARB) or develop in the environment as a result of direct uptake of antibiotic-resistant genes (ARG),

proliferation under environmental selection caused by antibiotics and co-selecting agents such as biocides, toxic metals, and nanomaterial stressors (Ashbolt *et al.*, 2013). According to results obtained in the current study, mock occurrences could be used to assess the potency of known compounds/mixtures, consequences of critical exposure levels and growth and persistence of dangerous strains (Ashbolt *et al.*, 2013). Where possible such assessments should be performed in open environments, rather than microcosms. This would take into account the variation of the chemicals in rivers during the seasons and the resident microbial community.

We provide an example of data acquisition for the integration of the assessment: replication of resistant bacteria was found to be reduced in the presence of phenanthrene, a known pollutant, and increased in presence of iron. The overall growth of an organism is the balance resulting from the sum of each single factor. Reduction of diversification of expressed protein is an indication of stress. The differential proteome analysis can support the identification of key proteins providing additional tools for bioindicators.

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CAPTIONS

Table 1. Concentrations of heavy metals in different location of River Thames.

Table 2. Relative gene expression of *S. flexneri tetR* and *tetA* on treated and untreated Thames water and M9 medium microcosms.

607 **Table 3.** Concentration of tetracycline degraded in *S. flexneri* 2a YSH6000 culture in
608 downstream water microcosm containing $FeCl_3$.
609
610 **Figure 1.** Proliferation of *Shigella flexneri* 2a YSH6000 in River Thames water microcosms in
611 the presence of different concentrations of oxytetracycline in neat and diluted water. Panel A.
612 Growth of *S. flexneri* at different concentrations of oxytetracycline ranging from 10 to 360
613 µg/mL. Panel B. Growth of *S. flexneri* at different fold dilutions of River Thames water. Error
614 bars represent standard error.
615
616 **Figure 2.** Growth of *S. flexneri* 2a YSH6000 in presence of phenanthrene with (A) and without
617 (B) 160 ug/mL oxytetracycline in M9 medium. Asterisk represents significant difference at
618 $p<0.05$. Error bars represent standard error.
619
620 **Figure 3.** Growth curves of *S. flexneri* 2a YSH6000 in presence of polyaromatic hydrocarbon
621 with and without (w/o) oxytetracycline. Pyrene (A and B); anthracene (C and D);
622 benzo(a)pyrene (E and F); and benzo(a,)anthracene: (G and H). Error bars represent standard
623 error.
624
625 **Figure 4.** Growth of *S. flexneri* 2a YSH6000 in River Thames water microcosms in presence
626 of different concentrations of phenanthrene with and without oxytetracycline. Growth of *S.*
627 *flexneri* in upstream River Thames water microcosms with phenanthrene (1.10 µg/L is the
628 measured concentration of the upstream water). Measurements were performed at 0 , 7.5,
629 and 13.5 h. Panel A. Growth without oxytetracycline, no significant differences ($p<0.05$) were
630 detected. Panel B. Growth in presence of oxytetracycline. Asterisk represents significant
631 difference at $p<0.05$. Error bars represent standard error.
632
633 **Figure 5.** Proliferation of *S. flexneri* 2a YSH6000 in microcosms with upstream and
634 downstream River Thames microcosms. The river water was enriched with different

concentrations of $FeCl_3$ and exposed to 160 µg/mL oxytetracycline. Panel A, comparison of *Shigella* CFU/mL measured in presence of 6.48 mg/L and 3.15 mg/L in downstream and upstream microcosms, respectively. Panel B, comparison of *Shigella* CFU/mL measurement in presence of 6.48 mg/L in both downstream and upstream. Asterisk represents significant difference at $p<0.05$. Error bars represent standard error.

Figure 6. Global expression intensity map. Heat map showing upregulation (red) and downregulation (green) of proteins in *S. flexneri* having significantly regulated proteins with ≥ 2 matched peptides upon exposure to oxytetracycline. Panel A. *S. flexneri* differential proteome in upstream river sector. Panel B. *S. flexneri* differential proteome from samples taken from the downstream river sector.

Figure 7. Global expression intensity map. Heat map showing upregulation (red) and downregulation (green) of proteins in *S. flexneri* having significantly regulated proteins with ≥ 2 matched peptides with and without exposure to oxytetracycline. Panel A. Differential detection of proteins of *S. flexneri* downstream sector versus upstream. Panel B. *S. flexneri* differential proteome in downstream river sector microcosms in presence of oxytetracycline.

Supplementary material 1. Primers used in this study

Supplementary material 2. List of the proteins detected in the differential proteomic study.