

Middlesex University Research Repository

An open access repository of
Middlesex University research

<http://eprints.mdx.ac.uk>

Maruzani, Rugare, Pathak, Ayush, Ward, Malcolm, Serafim, Vlad, Pantoja Munoz, Leonardo, Shah, Ajit J. ORCID logoORCID: <https://orcid.org/0000-0002-2350-6384> and Marvasi, Massimiliano ORCID logoORCID: <https://orcid.org/0000-0001-8525-7232> (2020) Antibiotic selective pressure in microcosms: pollution influences the persistence of multidrug resistant *Shigella flexneri* 2a YSH6000 strain in polluted river water samples. *Environmental Technology and Innovation*, 19 , 100821. ISSN 2352-1864 [Article] (doi:10.1016/j.eti.2020.100821)

Final accepted version (with author's formatting)

This version is available at: <https://eprints.mdx.ac.uk/29725/>

Copyright:

Middlesex University Research Repository makes the University's research available electronically.

Copyright and moral rights to this work are retained by the author and/or other copyright owners unless otherwise stated. The work is supplied on the understanding that any use for commercial gain is strictly forbidden. A copy may be downloaded for personal, non-commercial, research or study without prior permission and without charge.

Works, including theses and research projects, may not be reproduced in any format or medium, or extensive quotations taken from them, or their content changed in any way, without first obtaining permission in writing from the copyright holder(s). They may not be sold or exploited commercially in any format or medium without the prior written permission of the copyright holder(s).

Full bibliographic details must be given when referring to, or quoting from full items including the author's name, the title of the work, publication details where relevant (place, publisher, date), pagination, and for theses or dissertations the awarding institution, the degree type awarded, and the date of the award.

If you believe that any material held in the repository infringes copyright law, please contact the Repository Team at Middlesex University via the following email address:

eprints@mdx.ac.uk

The item will be removed from the repository while any claim is being investigated.

See also repository copyright: re-use policy: <http://eprints.mdx.ac.uk/policies.html#copy>

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

3
4 *Rugare Maruzani*¹, *Ayush Pathak*¹, *Malcolm Ward*¹, *Vlad Serafim*¹, *Leonardo Pantoja*
5 *Munoz*¹, *Ajit J Shah*¹, *Massimiliano Marvasi*^{2*}

6
7 ¹ Middlesex University London, Department of Natural Sciences, London. United Kingdom.

8 ² University of Florence, Department of Biology, Florence. Italy.

9
10 * Corresponding Author: Massimiliano Marvasi, University of Florence, Via Madonna del Piano
11 6. Sesto Fiorentino, Florence, Italy. Email: massimiliano.marvasi@unifi.it. Tel +39 055
12 4574749.

13
14
15 **ABSTRACT**

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

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

35

36

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

39

40

41

42

43

44 1. INTRODUCTION

45

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

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

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

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

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

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

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

98

99

100 **2. MATERIALS AND METHODS**

101

102 *2.1 Sampling sites.* River water was sampled at two locations of the River Thames (London,
103 UK): upstream and downstream of the city centre (geospatial coordinates are available
104 (Maruzani *et al.*, 2018)). Sampling sector were approximately 40 km apart . For each sampling
105 point, three 2 L samples were collected in different parts of the river. The sampling was
106 performed by sampling water in polyethylene terephthalate bottles. The samples were frozen
107 within 7 h of sampling.

108

109 *2.2 Strain used in this study.* The strain used in this study is *Shigella flexneri* 2a YSH6000
110 (Rajakumar, Sasakawa and Adler, 1996). It harbours a 66 Kbps resistance locus pathogenicity
111 island (SRL PAI) containing a 16 Kbps SRL region which encodes for resistance to
112 streptomycin (*aadA1*), ampicillin (*oxa-1*), chloramphenicol (*cat*) and tetracycline (*tetRA* - efflux
113 pump and receptor) (Sasakawa *et al.*, 1986). The bacterium was cultured overnight in
114 Lysogeny Broth (LB) medium (Oxoid, Basingstoke, UK), or in 1 X Minimal Salt (M9 medium)
115 (Invitrogen, Carlsbad, US). M9 medium was prepared according to manufacturer's
116 specifications and enriched with 12.5 µM nicotinic acid (Sigma-Aldrich, Dorset, UK) (*S. flexneri*
117 2a YSH6000 is auxotroph for nicotinic acid) and 0.2 % w/v of glucose (Sigma-Aldrich, Dorset,
118 UK) to produce the final M9 medium. Glucose (0.2 %, w/v) was added to strains that were
119 grown in filtered River Thames water in order to support growth.

120

121 *2.3 Filtration of the River Thames water samples.* Aliquots (200 mL) of water from each
122 sampling site were filtered twice using Whatman paper No 1 (particle retention 11 µm) (Sigma-
123 Aldrich, Dorset, UK) then twice using 0.22 µm filters (Billerica, MA, USA). Subsequently, the
124 three samples from the same river sector were combined to obtain "upstream" and
125 "downstream" samples. These were subsequently aliquoted into 50 mL Falcon tubes (Fisher,
126 Basingstoke, UK) and frozen at -20 °C until analysis.

127

128 *2.4 Degradation of tetracycline.* M9 overnight culture of *S. flexneri* was washed three times
129 with fresh M9 medium or River Thames water before being used as inoculum. A small volume
130 (30 µL) of 10⁸ cell/mL (the initial inoculum was estimated via serial dilutions and CFU counting
131 on LB plate) was inoculated into 2 mL of M9 medium or River Thames (enriched with 0.2 %
132 w/v of glucose) with 1.5 µg/L of oxytetracycline (Sigma-Aldrich, Dorset, UK). The microcosms
133 were incubated at 30 °C for up to 72 h without shaking. The microcosms were then filtered
134 using 0.22 µm filter (Billerica, MA, USA). The concentration of oxytetracycline in the filtrate
135 was measured using high performance liquid chromatography with tandem mass
136 spectrometry. The instrumentation and conditions that were used for measurement of

137 oxytetracycline were identical to those reported previously (Maruzani *et al.*, 2018). Briefly, an
138 X-LC UHPLC system (Jasco, Great Dunmow, UK) coupled to an API 3000 triple quadrupole
139 mass spectrometer (Applied Biosystems, Warrington, UK) was used. The software used to
140 acquire data and set the instrument parameters was Analyst version 1.4.2 (Applied
141 Biosystems, Warrington, UK). The chromatographic separation was achieved using a
142 Onyxmonolithic C18 100 x 3.0 mm i.d. connected to a 5 x 3.0 mm guard cartridge containing
143 the same stationary phase (Phenomenex, Macclesfield, UK). A linear binary gradient
144 composed of A - 0.1 % formic acid in water and B - 0.1 % formic acid in acetonitrile was used.
145 The elution profile used started at 10 % B then increased to 75 % over 5 min and was held at
146 this level for 1 min. The flow rate was set at 0.21 mL/min. An injection volume of 10 μ L was
147 used and the column oven temperature was maintained at 50°C. The MS was equipped with
148 an electrospray ionisation source which was operated in the positive-ion mode. The MRM
149 transitions for oxytetracycline of m/z 445.3 \rightarrow 410.1 and 445.3 \rightarrow 154.5 were monitored
150 simultaneously. The detection limit (LOD) was established as the lowest concentration of the
151 calibration standard that was detected with a signal-to-noise (S/N) ratio \geq 3:1 while the
152 quantification limit (LOQ) was established as the lowest concentration of the calibration
153 standard that was detected with a signal-to-noise (S/N) ratio \geq 10:1. The LOD and LOQ were
154 2 and 10 ng/mL respectively. The retention time of the antibiotic was found to be 3.1 min.
155 Oxytetracycline was identified by retention times (Rt) and by two selected reaction monitoring
156 (SRM) transitions.

157

158 *2.5 Heavy metal analysis of River Water.* Quantitation was performed using an ICP-MS (X
159 series II, Thermo Scientific, UK) using a 20 μ g L⁻¹ indium as an internal standard. The
160 instrument was tuned daily using a 10 μ g/L⁻¹ mixture of indium, cerium, cobalt, uranium and
161 lithium solution. All dilutions and external calibration standards were prepared using deionised
162 water with a conductivity of 18.2 M Ω obtained from a Purite water purification system (Thame,
163 UK). Magnesium, potassium, lithium, manganese, iron, copper, zinc, indium and cadmium
164 solutions and nitric acid (trace metal analysis) were obtained from Fisher Scientific

165 (Loughborough, UK). Method quantification limits (MQL) were calculated by multiplying the
166 standard deviation signal by 10 (at the retention time of the quantification species) of seven
167 consecutive blank samples. Method detection limits (MDL) were calculated by multiplying the
168 standard deviation by 3.14 times the signal of seven consecutive blank samples to achieve 99
169 % confidence intervals (CI). PlasmaLab software (ThermoFisher Scientific, Hemel Hempsted,
170 UK) was used to set instrument parameters and analyse the data.

171

172 *2.6 Proliferation of Shigella flexneri in River Thames microcosms.* Aliquots (200 μ L) of filtered
173 water from upstream and downstream (enriched with 0.2% w/v glucose) was added to a sterile
174 96-well microplate. Each well was inoculated with approximately 200-500 cells of *S. flexneri*
175 (the initial inoculum was estimated via serial dilutions and CFU counting on LB plate). The
176 plate was incubated at 30 °C for 48 h. After incubation, 25 μ L of sample from each well was
177 spread onto LB plate or Hektoen Enteric Agar (to confirm absence of contamination) and
178 log(CFU/mL) was calculated for the CFU obtained from LB agar plates. *S. flexneri* proliferation
179 was also measured in River Thames non diluted (ND), as control, and diluted 1:2 and 1:20
180 using distilled water enriched with 0.2% (v/v) sterile glucose to support growth. CFUs were
181 obtained as described above.

182 For the experiments with iron, *S. flexneri* was again cultured in the presence of FeCl₃ (Fisher
183 Scientific, Leicestershire, UK), the iron was added to the wells at a final concentration of 6.48
184 mg/L. The experiments were carried out in presence of 160 μ g/mL oxytetracycline. Growth was
185 measured by counting CFUs as described above. Experiments were carried out with at least
186 three biological and two technical replicates.

187

188 *2.7 Growth with oxytetracycline and PAHs.* Small volumes (200 μ L) of M9 medium was added
189 to sterile 96-well microplate and enriched with 0.2% (w/v) sterile glucose and 12.5 μ M nicotinic
190 acid to support growth. When appropriate, 160 μ g/mL of oxytetracycline and 140 μ g/L each of
191 phenanthrene, dibenz(a,h)anthracene, pyrene, anthracene, benzo(a)pyrene (Sigma-Aldrich
192 Dorset, UK) were added. Each well was initially inoculated with approximately 200-500 cells

193 of *S. flexneri*. The plates were incubated in a microplate reader GMB LabTech set at 30 °C
194 with absorbance measured using OD=600 nm at 0, 7 and 13.5 h. For experiments that were
195 repeated with upstream and downstream River Thames samples, water was enriched with
196 140.1 µg/L phenanthrene. Control wells with no phenanthrene were included here. It has to
197 be noted that in upstream water the basal level of phenanthrene was 1.1 µg/L (Maruzani *et*
198 *al.*, 2018). The study included three biological and two technical replicates.

199

200 *2.8 qPCR to verify the expression of tetAR genes in S. flexneri.* One (1) mL from overnight
201 cultures of *S. flexneri* in M9 medium was centrifuged and pellet was washed three times and
202 the pellet was re-suspended in 4 mL of upstream, downstream or M9 medium. All microcosms
203 were enriched with glucose and nicotinic acid. Oxytetracycline (160 µg/mL) was added when
204 appropriate. Controls did not contain oxytetracycline. Microcosms were incubated at 30 °C
205 without shaking for 48 h. Total RNA was extracted from samples using mirVana™ miRNA
206 Isolation Kit (Life Technologies, Carlsbad, CA, USA) using the manufacturers' instructions.
207 RNA integrity was visualized on 1.3 % agarose gel electrophoresis. Samples were quantified
208 using a Nanodrop spectrophotometer (ThermoFisher Scientific, Loughborough, UK) in
209 accordance with the manufacturers' instructions. DNA was degraded using TURBO DNA-
210 free™ Kit (Life Technologies). cDNA synthesis was performed using a
211 qPCRBIO cDNA Synthesis Kit (PCRBio) in accordance with the user manual guide using
212 random hexamer primers. qPCR was performed on a qPCR LightCycler 96 System (Life
213 Technologies, Carlsbad, CA, USA) by using PCRBIO SyGreen Mix Hi-ROX (PCR Biosystems,
214 London, UK). A negative control was obtained by using PCR grade water in place of a cDNA
215 template. DNA-free RNA was tested via standard PCR amplification to ensure complete
216 removal of genomic DNA prior to cDNA generation by using 16S primers (Marvasi *et al.*, 2009).
217 *Shigella acnB* gene was used as an internal reference gene. qPCR was performed by using
218 the following cycles: initial denaturation at 95°C for 2 min, 40 cycles of denaturation at 95°C,
219 annealing at 60°C and extending at 65°C for 30 s. Primers used in PCR reactions are shown
220 in Supplementary Material S2. Minimum requirement tests were carried out to ensure specific

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

225

226 **2.9 Comparative proteome analysis.** An overnight *S. flexneri* strain grown in M9 medium was
227 diluted until OD₆₀₀ was 0.20 absorbance units. A small volume (1 mL) was inoculated into 6
228 mL of microcosms containing upstream, downstream water and M9 enriched with 0.2% w/v
229 glucose. In parallel, the same microcosms were treated with 160 µg/mL of oxytetracycline.
230 The microcosms were incubated for 8 h at 30 °C in a static incubator. Tubes were centrifuged
231 and cells were re-suspended in sterile 50 mM EDTA. The cells were sonicated at amplitude
232 7.2 with 6 cycles of 30 s each, with 1 min in ice between the cycles. Cellular fragmentation
233 was verified using a microscope. Cell debris was removed by centrifuging samples at 13,000
234 x g for 10 min. The supernatant was mixed with 4 volumes of cold acetone and then left for 1
235 h at -20 °C. After incubation, the samples were centrifuged at 13,000 x for 10 min. Supernatant
236 was discarded, and the pellet was dried and re-suspended in 50 mM EDTA. Proteins were
237 then reduced, alkylated and subsequently digested using 2 µL of trypsin (0.1 µg/µL dissolved
238 in 50 mM ammonium bicarbonate) at 37 °C for 3 h. A further 2 µL of trypsin was added to the
239 samples and the mixture was incubated at 37 °C for an additional 2 h. The samples were
240 diluted in 150 µL 50 mM ammonium bicarbonate and passed through Mini-Uni Prep filter
241 devices.

242 **Mass Spectrometry.** Tryptic digests were analysed using a Dionex Ultimate 3000 RSLC Nano
243 ultra-high performance liquid chromatography system coupled to a Q Exactive mass
244 spectrometer. Aliquot (15 µL) of tryptic digest was desalted and concentrated using a 5 mm x
245 300 µm i.d. C18 trap cartridge and solvents composed of a mixture of water and acetonitrile
246 (98:2 %, v/v) containing 0.05 % TFA (loading solvent A) and a mixture of acetonitrile and water
247 (80:20 %, v/v) (loading solvent B) at a flow rate of 20 µL/min. The concentrated sample was
248 separated using a binary gradient elution profile composed of a mixture of water and

249 acetonitrile (95:5,v/v) containing 0.1 % formic acid (eluent A) and a mixture of acetonitrile and
250 water (80:20 %, v/v) (eluent B) containing 0.1 % formic acid at a flow rate of 6 μ L/min. The
251 gradient was 0 min- 0 %B, 4 min- 5 %B, 5 min- 8 %B, 40 min-40 %B, 41 min-80 %B. The
252 autosampler and column oven temperature was set to 4 and 40 °C respectively. The Q
253 Exactive was operated in a data dependent mode. MS survey scans were acquired from m/z
254 350 to 2000 at resolution of 70,000 with AGC of 3e6 and maximum IT of 100 ms. The 20 most
255 abundant ions were subjected to MS/MS and measured with a resolution of 17,000 and AGC
256 of 1e5 and maximum IT of 50 ms.

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

264

265 *2.10 Statistical analysis.* T-tests, Anova or Tukey's mean separation tests were performed by
266 using JMP statistical software (SAS Institute, Cary, NC, USA). Graphs were drawn using Prism
267 8.0.

268

269

270 **3. RESULTS**

271

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

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

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

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

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

298

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

300 Downstream river water contains a complex mixture of thousands of molecules, we focused
301 our attention on six chemicals: phenanthrene, pyrene, anthracene, benzo(a)pyrene and
302 benzo(a)anthracene (and iron (Table 1)). To study the effect of each compound in association
303 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 (FeCl₃) 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 FeCl₃ was added. No
327 degradation of oxytetracycline was observed in the presence of 6 and 12 mg/L of FeCl₃ over
328 3 days (Table 3).

329

330

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

333

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

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

350 When *S. flexneri* cells were exposed to downstream water with and without oxytetracycline,
351 only three proteins were significantly differentially regulated in presence of tetracycline (Figure
352 6 B): a 30S large ribosomal subunit [B11PY8], a S-ribosyl homocysteine lyase [B0TR04] of
353 *Shewanella halifaxensis* (a gamma-proteobacteria), used to communicate both the cell
354 density and the metabolic potential of the environment. The third proteins is a cold shock-like
355 protein CspC [P0A9Y7].

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

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

375

376

377 **4. DISCUSSION**

378

379 Environmental risk assessment in terms of selection for antimicrobial resistance is not
380 performed because there are no ecotoxicological assays with selective endpoints to determine
381 the concentrations of antibiotics that select for AMR (Murray, Stanton and Zhang, 2019).
382 Therefore, the study of mock occurrences of lethal concentrations in river water (or other
383 environments) can provide useful information about the fate of a strain or antibiotics in a
384 specific environmental context. There are at least three important reasons for carrying out
385 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

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

423 On the other hand, iron was found to promote growth. It has been shown that Fe(III) ions in
424 aqueous solution in the absence of light induced degradation of three tetracycline antibiotics,
425 tetracycline, oxytetracycline and chloro-oxytetracycline (Wang *et al.*, 2015). Also, Wang *et al.*
426 [21] demonstrated that Fe(II) complexes with tetracycline, oxytetracycline, or chloro-
427 oxytetracycline which could simultaneously lead to the accelerated oxidation of Fe(II) to Fe(III)
428 and faster degradation of tetracyclines (Wang *et al.*, 2016). Although, the Fe(III) cannot be
429 oxidised further, in some cases it can be reduced to Fe(II) again by tetracyclines (Wang *et al.*,
430 2016), supporting the role of chemicals in the environment that ultimately leads to the
431 equilibrium of Fe(II)/Fe(III). However, in our experimental setting, oxytetracycline was not
432 degraded in the presence of iron Fe(III) within 3 days at 30°C (Table 3), showing the
433 importance of the chemical environment in supporting or preventing these redox reactions.
434 Because River Thames is a complex environment, we also found that other organic molecules
435 can compete and chelate with iron, for example humic acids. These are present in River
436 Thames and can compete with tetracyclines for Fe(II) (Wang *et al.*, 2016; Watt *et al.*, 1996;
437 Old *et al.*, 2019) in the open system. Ultimately, the current study has shown that excess of
438 iron may foster growth of resistant bacteria even in presence of selective pressure.

439

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

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

452 In the absence of any selective pressure a comparison of organism cultured in upstream
453 versus downstream microcosms showed 64 proteins that are differentially regulated. In our
454 experiment, exposure of *S. flexneri* to oxytetracycline appeared to repress protein transcription
455 which suggests a response to stress conditions and the toxic effect of oxytetracycline. Some
456 proteins have no benefits in an environment incurring a quantifiable fitness costs on cellular
457 growth rates. Certainly, the magnitude and variability of unused protein expression is largely
458 due to the challenge in determining environment-specific proteome utilisation, and unused
459 protein abundances are not constant across environments (O'Brien, Utrilla and Palsson,
460 2016). In our study, the addition of oxytetracycline to any tested environment (upstream,
461 downstream and M9 medium) reduced the number of differently regulated proteins. Reduction
462 of proteins accounts for slow replication and it is known that slower growing cells are more
463 resistant to cellular stresses, such as response to antibiotics (Poole, 2012; O'Brien, Utrilla and
464 Palsson, 2016).

465

466 **Conclusions**

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

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

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

484

485 **ACKNOWLEDGMENTS**

486 We thank Thames21 (UK) and volunteers for sampling the Thames water. We are grateful to
487 Manika Choudhury for the technical support.

488

489

490 **REFERENCES**

491

- 492 Adebusuyi, A. A. and Foght, J. M., 2011. An alternative physiological role for the EmhABC
493 efflux pump in *Pseudomonas fluorescens* cLP6a. BMC Microbiol. 11, 252.
494 <https://doi.org/10.1186/1471-2180-11-252>
- 495 Adebusuyi, A. A., Smith, A. Y., Gray, M. R. and Foght, J. M., 2012. The EmhABC efflux pump
496 decreases the efficiency of phenanthrene biodegradation by *Pseudomonas fluorescens*

497 strain LP6a. *Appl. Microbiol. Biotechnol.* 95(3), 757-766. <https://doi.org/10.1186/1471->
498 2180-11-252

499 Ashbolt, N. J., Amézquita, A., Backhaus, T., Borriello, P., Brandt, K. K., Collignon, P., Coors,
500 A., Finley, R., Gaze, W. H., Heberer, T., Lawrence, J. R., Larsson, D. G., McEwen, S.
501 A., Ryan, J. J., Schönfeld, J., Silley, P., Snape, J. R., Van den Eede, C. and Topp, E.,
502 2013. Human Health Risk Assessment (HHRA) for environmental development and
503 transfer of antibiotic resistance. *Environ. Health Perspect.* 121(9), 993-1001.
504 <https://doi.org/10.1289/ehp.1206316>

505 Baveja, U. K., 2014. Shigellosis: An emerging water-related public health problem. In: Singh
506 P., Sharma V. (eds) *Water and Health*. Springer, New Delhi pp. 107-117.

507 Boxall, A. (2019) Identifying hotspots of resistance selection from antibiotic exposure in urban
508 environments around the World. Europe 29th Annual Meeting Helsinki: SETAC.

509 Brack, W. and Schulze, T., 2019. Risk-based prioritisation of organic micropollutants in WWTP
510 effluents and surface waters on a European scale. Europe 29th Annual Meeting,
511 Helsinki.

512 Brown, P. C., Borowska, E., Schwartz, T. and Horn, H., 2019. Impact of the particulate matter
513 from wastewater discharge on the abundance of antibiotic resistance genes and
514 facultative pathogenic bacteria in downstream river sediments. *Sci. Tot. Env.* 649, 1171-
515 1178. <https://doi.org/10.1016/j.scitotenv.2018.08.394>

516 Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R.,
517 Nolan, T., Pfaffl, M. W., Shipley, G. L., Vandesompele, J. and Wittwer, C. T., 2009. The
518 MIQE Guidelines: Minimum information for publication of quantitative real-time PCR
519 experiments. *Clinic. Chem.* 55(4), 611. <https://doi.org/10.1373/clinchem.2008>

520 Di Cesare, A., Fontaneto, D., Doppelbauer, J. and Corno, G., 2016. Fitness and recovery of
521 bacterial communities and antibiotic resistance genes in urban wastewaters exposed to
522 classical disinfection treatments. *Env. Sci. Technol.* 50(18), 10153-10161.
523 <https://doi.org/10.1021/acs.est.6b02268>

524 EMA (2018) Guideline on the assessment of the risk to public health from antimicrobial
525 resistance due to the use of an antimicrobial veterinary medicinal product in food
526 producing animals. European Medicines Agency. London

527 Group, S., 2005. Thames tideway strategic study. Doc Ref: 8.1.2 Steering Group Report
528 Report). Thames Tideway Tunnel, Thames Water Utilities Limited.

529 Hanamoto, S., Nakada, N., Jürgens, M. D., Johnson, A. C., Yamashita, N. and Tanaka, H.,
530 2018. The different fate of antibiotics in the Thames River, UK, and the Katsura River,
531 Japan. *Env. Sci. Poll. Res.* 25(2), 1903-1913. [https://doi.org/10.1007/s11356-017-0523-](https://doi.org/10.1007/s11356-017-0523-z)
532 [z](https://doi.org/10.1007/s11356-017-0523-z)

533 Hong, P.-Y., Julian, T. R., Pype, M.-L., Jiang, S. C., Nelson, K. L., Graham, D., Pruden, A.
534 and Manaia, C. M., 2018. Reusing treated wastewater: consideration of the safety
535 aspects associated with antibiotic-resistant bacteria and antibiotic resistance genes.
536 *Water.* 10(3), 244-244. <https://doi.org/10.3390/w10030244>

537 Jones-Dias, D., Carvalho, A. S., Moura, I. B., Manageiro, V., Igrejas, G., Caniça, M. and
538 Matthiesen, R., 2017. Quantitative proteome analysis of an antibiotic resistant
539 *Escherichia coli* exposed to tetracycline reveals multiple affected metabolic and
540 peptidoglycan processes. *J. Proteomics.* 156, 20-28.
541 <https://doi.org/10.1016/j.jprot.2016.12.017>

542 Maruzani, R., Canali, A., Serafim, V., Munoz, L. P., Shah, A. J., Perito, B. and Marvasi, M.,
543 2018. Effect of anthropogenic pollution on the fitness of tetracycline sensitive *Shigella*
544 *flexneri* in Thames river water. *J. Env. Chem. Eng.* 6(1), 19-27.
545 <https://doi.org/10.1016/j.jece.2017.11.069>

546 Marvasi, M., Vedovato, E., Balsamo, C., Macherelli, A., Dei, L., Mastromei, G. and Perito, B.,
547 2009. Bacterial community analysis on the Mediaeval stained glass window “Natività” in
548 the Florence Cathedral. *J. Cult. Herit.* 10(1), 124-133.
549 <https://doi.org/10.1016/j.culher.2008.08.010>

550 Moller, T. S. B., Overgaard, M., Nielsen, S., Bortolaia, V., Sommer, M. O. A., Guardabassi, L.
551 and Olsen, J. E., 2016. Relation between *tetR* and *tetA* expression in tetracycline

552 resistant *Escherichia coli*. BMC Microbiol. 16(1), 39. <https://doi.org/10.1186/s12866->
553 016-0649-z

554 Murray, A., Stanton, I. and Zhang, L., 2019. A novel method for determining effect
555 concentrations that select for antimicrobial resistance. Europe 29th Annual Meeting -
556 Abstract Book, Helsinki: SETAC

557 O'Brien, E. J., Utrilla, J. and Palsson, B. O., 2016. Quantification and classification of *E. coli*
558 proteome utilization and unused protein costs across environments. PLoS Comput. Biol.
559 12(6), e1004998. <https://doi.org/10.1371/journal.pcbi.1004998>

560 Old, G. H., Naden, P. S., Harman, M., Bowes, M. J., Roberts, C., Scarlett, P. M., Nicholls, D.
561 J. E., Armstrong, L. K., Wickham, H. D. and Read, D. S., 2019. Using dissolved organic
562 matter fluorescence to identify the provenance of nutrients in a lowland catchment; the
563 River Thames, England. Sci. Tot. Env. 653, 1240-1252.
564 <https://doi:10.1016/j.scitotenv.2018>

565 Poole, K., 2012. Stress responses as determinants of antimicrobial resistance in Gram-
566 negative bacteria. Trends Microbiol. 20(5), 227-34.
567 <https://doi:10.1016/j.tim.2012.02.004>

568 Rajakumar, K., Sasakawa, C. and Adler, B., 1996. A spontaneous 99-kb chromosomal
569 deletion results in multi-antibiotic susceptibility and an attenuation of contact haemolysis
570 in *Shigella flexneri* 2a. J. Med. Microbiol. 45(1), 64-75.

571 Sasakawa, C., Kamata, K., Sakai, T., Murayama, S. Y., Makino, S. and Yoshikawa, M., 1986.
572 Molecular alteration of the 140-megadalton plasmid associated with loss of virulence
573 and Congo red binding activity in *Shigella flexneri*. Infect. Immun. 51(2), 470-5.

574 Sims, G. E. and Kim, S. H., 2011. Whole-genome phylogeny of *Escherichia coli*/*Shigella* group
575 by feature frequency profiles (FFPs). Proc. Natl. Acad. Sci. USA. 108(20), 8329-34.
576 <https://doi.org/10.1073/pnas.1105168108>

577 Vranakis, I., De Bock, P. J., Papadioti, A., Tselentis, Y., Gevaert, K., Tsiotis, G. and Psaroulaki,
578 A., 2012. Quantitative proteome profiling of *C. burnetii* under tetracycline stress
579 conditions. PLoS One. 7(3), e33599. <https://doi:10.1371/journal.pone.0033599>

- 580 Wang, H., Yao, H., Sun, P., Li, D. and Huang, C. H., 2016. Transformation of tetracycline
581 antibiotics and Fe(II) and Fe(III) species induced by their complexation. *Env. Sci.*
582 *Technol.* 50(1), 145-53. <https://doi.org/10.1021/acs.est.5b03696>
- 583 Wang, H., Yao, H., Sun, P., Pei, J., Li, D. and Huang, C.-H., 2015. Oxidation of tetracycline
584 antibiotics induced by Fe(III) ions without light irradiation. *Chemosphere.* 119, 1255-
585 1261. <https://doi.org/10.1016/j.chemosphere.2014.09.098>
- 586 Wang, J., Zhao, Z., Chen, J., Lu, H., Liu, G., Zhou, J. and Guan, X., 2017. PAHs accelerate
587 the propagation of antibiotic resistance genes in coastal water microbial community.
588 *Env. Poll.* 231, 1145-1152. <https://doi:10.1016/j.envpol.2017.07.067>
- 589 Watt, B. E., Malcolm, R. L., Hayes, M. H. B., Clark, N. W. E. and Chipman, J. K., 1996.
590 Chemistry and potential mutagenicity of humic substances in waters from different
591 watersheds in Britain and Ireland. *Water Res.* 30(6), 1502-1516.
592 [https://doi.org/10.1016/0043-1354\(95\)00319-3](https://doi.org/10.1016/0043-1354(95)00319-3)
- 593 Xu, C., Lin, X., Ren, H., Zhang, Y., Wang, S. and Peng, X., 2006. Analysis of outer membrane
594 proteome of *Escherichia coli* related to resistance to ampicillin and tetracycline.
595 *Proteomics.* 6(2), 462-473. <https://doi.org/10.1002/pmic.200500219>
- 596 Zijp, M. C., Posthuma, L. and van de Meent, D., 2014. Definition and applications of a versatile
597 chemical pollution footprint methodology. *Env. Sci. Technol.* 48(18), 10588-10597.
598 <https://doi.org/10.1021/es500629f>

599

600

601 CAPTIONS

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

603

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

606

607 **Table 3.** Concentration of tetracycline degraded in *S. flexneri* 2a YSH6000 culture in
608 downstream water microcosm containing FeCl₃.

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 µg/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

635 concentrations of FeCl₃ and exposed to 160 µg/mL oxytetracycline. Panel A, comparison of
636 *Shigella* CFU/mL measured in presence of 6.48 mg/L and 3.15 mg/L in downstream and
637 upstream microcosms, respectively. Panel B, comparison of *Shigella* CFU/mL measurement
638 in presence of 6.48 mg/L in both downstream and upstream. Asterisk represents significant
639 difference at p<0.05. Error bars represent standard error.

640

641

642 **Figure 6. Global expression intensity map.** Heat map showing upregulation (red) and
643 downregulation (green) of proteins in *S. flexneri* having significantly regulated proteins with ≥
644 2 matched peptides upon exposure to oxytetracycline. Panel A. *S. flexneri* differential
645 proteome in upstream river sector. Panel B. *S. flexneri* differential proteome from samples
646 taken from the downstream river sector.

647

648 **Figure 7. Global expression intensity map.** Heat map showing upregulation (red) and
649 downregulation (green) of proteins in *S. flexneri* having significantly regulated proteins with ≥
650 2 matched peptides with and without exposure to oxytetracycline. Panel A. Differential
651 detection of proteins of *S. flexneri* downstream sector versus upstream. Panel B. *S. flexneri*
652 differential proteome in downstream river sector microcosms in presence of oxytetracycline.

653

654 **Supplementary material 1.** Primers used in this study

655

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

657