Effect of anthropogenic pollution on the fitness of tetracycline sensitive *Shigella flexneri* in Thames river water.

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ABSTRACT

Urban rivers may be source of antibiotics contamination that could support spread of antibiotic resistant bacteria (ARB) to the population. It is important to understand to what extent the presence of pollutants in urban rivers influences fitness of ARB. In an exercise to estimate this contribution, microcosms were generated from Thames river (London, UK) from different locations: upstream and downstream the city center. The concentration of the polycyclic aromatic hydrocarbons (PAHs) benzo(a)pyrene, pyrene and phenantrene was found to be 128, 171 and 128 times higher in downstream sector when compared to upstream sector, respectively. Filtered microcosms for each sector were enriched with tetracycline at lethal (10 µg/mL) and sub-lethal (10 ng/mL) concentrations and the fitness of an isogenic pair of *Shigella flexneri* 2a YSH6000 (tet^R)

and *S. flexneri* 2a 1363 (tet^S) was then measured. In the presence of selective pressure in upstream microcosms, the resistant strain outcompeted the sensitive one, as expected. In contrast, sensitive *S. flexneri* tet^S was found to significantly compete with resistant *S. flexneri* tet^R at lethal concentrations of tetracycline in downstream microcosms, where levels of PAHs were the highest. Further experiments showed that PAHs rendered the resistant *S. flexneri* tet^R ~20% more sensitive to tetracycline. Sensitive *S. flexneri* tet^S strain was able to persist at lethal concentration of tetracycline in downstream microcosms, at higher concentrations of PAHs. Our findings suggest that in a polluted river sensitive *S. flexneri* cells may still thrive in presence of selective pressure. Fitness tests provide an additional tool to measure bioavailability.

Key words: Antibiotic resistant bacteria (ARB), fitness test, tetracycline, river water, *Shigella flexneri,* PAHs, benzo(a)pyrene, pyrene, phenantrene

INTRODUCTION

Antibiotics are continuously released into the environment from human activities such as wastewater treatment plants and hospitals effluents, combined sewer overflows, processing plant effluents, application of agricultural waste and bio-solids to fields, leakage from waste-storage containers and landfills (Davies and Davies 2010; Michael et al. 2013). It is generally accepted that the presence of antibiotic residuals in the environment could exert a selective pressure supporting the spread of antibiotic resistance determinants through microbial communities (Knapp et al. 2010; Allen et al. 2013). In an urban river context the presence of antibiotic residuals is of great importance due to the risk that it exerts to the population. For example, recent measurements of tetracycline in urban rivers showed levels up to 5.4 µg/L and for

sediment in municipal biological wastewater treatment plants up to $1.6x10^2$ ng/g (Lu et al. 2015; Topal and Arslan Topal 2015; Xu et al. 2016; Dang et al. 2017). As a consequence, antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) have been isolated from water and urban rivers arguing for a possible correlation with the antiobiotic pollution (Xu et al. 2012; Zhang et al. 2015; Osinska et al. 2017). In addition, the use of disinfectants in urban wastewater treatment plants can promote a residual microbial community that is more resistant to antibiotics (Di Cesare et al. 2016). This is particularly dangerous in an urban context where the presence of high density population, where small wildlife and insects could potentially spread ARB to the population (Parker et al. 2016).

In some instances, antibiotic resistance has an energetic cost to the cell, therefore compensatory mutations are in place or the resistant bacteria may be subjected to reduced fitness when compared to the same strain without the resistance (Maisnier-Patin et al. 2002; Guo et al. 2012; Colicchio et al. 2015; Freihofer et al. 2016). When different resistance cassettes are considered, the fitness cost varies according to the antibiotic and the environment. For example tigecycline or tetracycline resistance comes with a substantial fitness cost (Linkevicius et al. 2013; Johnson et al. 2015). On the other hand, in the intestine of pigs, ampicillin resistant *Escherichia coli* have been shown not to carry a fitness cost for their resistance (Ahmad et al. 2016), nor fluoroquinolones resistance in *Salmonella enterica* serovar Typhi have been shown not to have a disadvantage over the sensitive parent strain (Baker et al. 2013). From these examples, it is clear that the environment plays a pivotal role in shaping the fitness of ARB, as the fitness is the organism's ability to survive and reproduce in its environment.

Even decades after the use of antibiotic became common, a complete picture of the ecology of the antibiotic resistant bacteria is missing. Minor knowledge is currently

available on to what extent the chemical environment affects the bioavailability of antibiotics in ecosystems (Sun et al. 2015). Moreover, it is unclear to what extent bioavailability would change the fitness of the antibiotic resistant bacteria favoring the proliferation of the resistant bacteria over the sensitive ones. Specifically, our research questions are addressed to study the correlation between the presence of tetracycline, PAHs and the fitness of ARB in polluted river water. A clearer understating of this would lead to the implementation of critical control points for managing the spread of antibiotic resistance cassettes and effective control at the tipping points. Specifically, our research questions are addressed to determine the fitness of bacteria in urban river waters enriched with sub-lethal and lethal concentrations of tetracycline. Microcosms from Thames river (London, UK) were used as environmental model of a highly populated metropolis in Europe. For this purpose we used microcosms, which are defined as simplified ecosystems that are used to study the behavior of a natural ecosystem under controlled conditions. As model organisms we used an isogenic pair of *Shigella flexneri* strains, resistant and sensitive to tetracycline, previously isolated from an outbreak (Reller et al. 2006). *S. flexneri* 2a strain naturally carries multi antibiotics resistance and it can infect at very low infection dose (tens of cells) (Baveja 2014). In recent years, multidrug resistance (MDR) in several *Shigella* strains has become a public health problem (Ashkenazi et al. 2003; Gupta et al. 2010). Genes harbored within the *Shigella* resistance locus pathogenicity island (SRL PAI) were identified as contributors to the resistance phenotype (Luck et al. 2001). The entire pathogenicity island is a 66 Kbps element that contains the 16 Kbps SRL region, which encodes for resistance to streptomycin (*aadA1*), ampicillin (*oxa-1*), chloramphenicol (*cat*) and tetracycline (*tetRA* - efflux pump and receptor) (Luck et al. 2001). Currently no studies have been reported that show a relationship between the presence of *S. flexneri* SRL island with survival of

104 the bacteria in the environment and its response to the antibiotics whilst reproducing in
105 such environment.
106 Our findings show that in an *in vitro* polluted river context (microcosms) the sensitive *S.*
107 *flexneri* cells may still have an advantage in presence of lethal concentration of
108 antibiotics and the competitive test provides a useful indication in terms of bioavailability
109 of the antibiotic tetracycline.
110
111
112 **MATERIALS AND METHODS**
113 *Sampling sites*. Thames river (London, UK) sampling sites were chosen in three different
114 sectors according to the river flow: upstream of the city center, city center, and
115 downstream of the city center (Figure 1). Each sampling sector was approximately 20
116 km apart from each other. For each sector, three 2 L samples were taken in different
117 parts of the river on September 2016 (Figure 1 and supplementary materials S1). These
118 were taken from the surface of the river using polyethylene terephthalate bottles and
119 frozen within 7 hours of sampling. All samples were transferred to the laboratory within 2
120 weeks for the generation of microcosms.
121 Samples from city center and downstream sectors were exposed to combined sewer
122 overflows (CSOs). CSOs release wastewater in the Thames when the water flow is
123 intense, eventually contaminating the river with untreated wastewater discharges
124 (Steering Group Report 2005; Schreiber et al. 2016)
125
126 *Strains used in this study.* Strains used in the competition analysis were the resistant
127 *Shigella flexneri* 2a YSH6000 (Rajakumar et al. 1996) (labeled as *S. flexneri* tet^R) and
128 sensitive *S. flexneri* 2a 1363 (labeled as *S. flexneri* tet^S), with a spontaneous deletion of
129 the SRL island (Luck et al. 2004). Strains were cultured overnight in LB medium (Oxoid,

130 Basingstoke, UK), or 1X Minimal Salt (M9 medium) (Invitrogen, Carlsbad, US). M9
131 medium was prepared according to manufacturer's specifications with 12.5 µM nicotinic
132 acid (Sigma-Aldrich St. Louis, MS, USA) (*S. flexneri* tet^R and *S. flexneri* tet^S are
133 auxotroph for nicotinic acid) and 0.2 % w/v of glucose (Sigma-Aldrich St. Louis, MS,
134 USA) were used to generate the M9 final medium.
135
136 *Water filtration.* 200 mL from each river sampling site were filtered twice using Whatman
137 paper No 1 (particle retention 11 µm) (Sigma-Aldrich St. Louis, MS, USA) then filtered
138 twice using 0.22 µm filters (Billerica, MA, USA) to ensure the removal of the microbial
139 community. The three samples from the same river sector were combined to form
140 "upstream", "center" and "downstream" samples. These were subsequently aliquoted
141 into 50 mL Falcon tubes (Fisher, Basingstoke, UK) and frozen at -20 ºC until analysis.
142
143 *Test for loss or acquisition of tetracycline cassette in S. flexneri strains*. We tested if the
144 tetracycline resistance carried by *S. flexneri* tet^R was persistent within a 1-week period.
145 Microcosms without selective pressure were prepared: i) A sample containing a mixture
146 of water from the three sectors of Thames and a control containing 0.85 % wt/v saline
147 solution were prepared. Microcosms were inoculated separately with 10^5 cells/mL of the
148 resistant bacterial strain and incubated at 30 ºC for 0, 2, 5 and 7 days. Following
149 incubation 100 CFU were picked and patched onto LB selective medium containing
150 tetracycline 10µg/mL.
151 To test horizontal tetracycline cassette acquisition, 10^5 cells/mL of the sensitive bacterial
152 strain were incubated in Thames water from three sectors mixed in equal proportions.
153 Cells were incubated for 48 hours at 30 ºC. After incubation 25 µL aliquots were plated
154 on LB plate supplemented with tetracycline at 10 µg/mL for the detection of resistant
155 colonies.

Three biological replicas for each microcosm were prepared.

Competitive test. To calculate the competitive index, overnight M9 cultures of *S. flexneri* tet^R and sensitive *S. flexneri* tet^S were washed three times with 9.89 g/L phosphate buffer saline (PBS) (Fisher, Basingstoke, UK) to remove the residual medium before inoculation. Washed cells were inoculated at 10^5 CFU/mL, at a 1:1 ratio normalized by measuring absorbance at OD_{595} using a UV/VIS spectrophotometer. To confirm that the 1:1 ratio was achieved, a sample of 200 colonies was immediately screened on LB selective medium (10 µg/mL tetracycline). Downstream microcosms were enriched with tetracycline at concentration of 10 ng/mL (sub-lethal) and 10 µg/mL (lethal) and no tetracycline for the negative control. Upstream microcosm was enriched with tetracycline at 10 µg/mL (lethal concentration). Microcosms were then incubated at 30 °C in sterile tubes which were kept static to simulate stagnant water. The tubes were opened daily under the BL2 cabinet for 10 minutes to allow gas exchanges and briefly shaken. At 0 and 48 hours aliquots were taken on LB agar plates and at least 50 colony-forming units (CFU) were picked and patched on selective LB medium containing 10 µg/mL tetracycline (Sigma-Aldrich St. Louis, MS, USA) in order to distinguish the resistant and sensitive cells. The ratio of the sensitive to resistant cells was calculated using the Competitive Index (CI) formula:

$$\log(Competitive\ Index) = \frac{R_{out}/S_{out}}{R_{in}/S_{in}}$$

Where:

R_{out} is the percentage of resistant at the day of sampling for each replica,

S_{out} is the percentage of sensitive at the day of sampling for each replica,

R_{in} is the percentage of resistant at the initial inoculum,

S_{in} is the percentage of sensitive at the initial inoculum.

For each microcosm 6 biological replicas and 50 colony forming units (CFU) for each replica were picked and patched on selective media.

Phage lysis test. 500 µL of overnight LB culture of *S. flexneri* tet^R and *S. flexneri* tet^S were resuspended in 20 mL of molten LB agar. Once plates had solidified, 0.5 mL of river water samples, ranging from undiluted up to 10^{-9} dilutions were spread above the surface. Plates were incubated at 30 ºC for up to one week. Plates were observed daily for a week to identify plaques of lysis. Three biological replicas were performed.

Chemical quantification of selected polycyclic aromatic hydrocarbons (PAHs). PAHs from samples were pre-concentrated and extracted using 6 mL C18 solid phase extraction cartridges, according to manufacturer's instructions (Thames Restek, Saunderton, UK). Briefly, cartridges were conditioned with 6 mL of methanol, equilibrated with 6 mL of deionized water. Subsequently, 100 mL of sample was loaded onto the cartridge. The cartridge was washed with 6 mL of a mixture of methanol:water (20:80, v/v) and then dried under full vacuum for 10 min. The PAHs were eluted with 6 mL of dichloromethane and then dried using a nitrogen evaporator. The sample was reconstituted in 1 mL of acetonitrile (Fisher Scientific UHPLC) on the day of analysis. PAHs used for preparing standards were purchased from the following companies: Phenanthrene (Sigma-AldrichPoole, UK,), pyrene (Acros organics, Fisher, Basingstoke, UK), benzo(a)pyrene (Alfa Aesar Haverhill, MA, USA).
Standards and extracts were analysed using a Waters Aquity UPLC system (Elstree, UK) composed of a quanternary puming system, an autosampler and a column oven. Separation was performed using a 2.1X150 mm Acquity UPLC BEH RP18 1.7 µm column (Waters, Elstree, UK) at a flow rate of 0.6 mL/min. A binary gradient elution

206 profile composed of solvent A: deionized water, B: acetonitrile, 0-4.5 min 50 % B, 4.5-5.5
207 min 67 % B, 5.5-7.5 min 67 %B, 7.5-9.5 min 77 %B, 9.5-12 min 77 %B, 12-12.5 50 % B,
208 12.5-17min 50 %B was used. An injection volume of 2 µL was used. Eluates were
209 monitored using a fluorescence detection with the excitation and emission wavelengths
210 set as indicated in Supplemental Material S2 (including the spectrum). External
211 calibration curves were prepared using PAH's at concentrations of 0, 2, 4, 6, 10 µg/L in
212 acetonitrile.
213
214 *Instrumentation and conditions used for measurement of tetracycline.* ALC-MS/MS
215 system was used for quantifying tetracycline. This consisted of an X-LC UHPLC system
216 (JASCO, UK) coupled to an API 3000 triple quadrupole mass spectrometer (Applied
217 Biosystems, Warrington, UK). The software used to acquire data and run the instrument
218 was Analyst version 1.4.2 (Applied Biosystems, Warrington, UK). The chromatographic
219 separation was achieved using an Ascentis Express C18 column (5 cm x 2.1 mm i.d.,
220 2.7 µm) from Sigma Aldrich (Poole, UK). A binary gradient of A - 0.1% formic acid in
221 water and B - 0.1 % formic acid in acetonitrile was used. The elution profile used started
222 at 10% B then increased at 75% over 5 min and maintained at this level for 1 min and
223 then returned to 10% B for 3 min to equilibrate the column. The flow rate was set at 0.21
224 mL/min. A volume of 10 µL was injected per run and the column oven temperature was
225 set at 50˚C. The MS electrospray source was operated in the positive-ion mode. The
226 MRM transitions for tetracycline *m/z* 445.3 → 410.1 and 445.3 → 154.5 were monitored
227 simultaneous. The detection limit (LOD) was established as the lowest concentration of
228 the calibration standard that was detected with a signal-to-noise (S/N) ratio ≥ 3:1 while
229 the quantification limit (LOQ) was established as the lowest concentration of the
230 calibration standard that was detected with a signal-to-noise (S/N) ratio ≥ 10:1. LOD and

231 LOQ were 2 and 10 ng/mL respectively. The retention time was 3.1 min. Tetracycline
232 was identified by retention times (Rt) and by 2 selected reaction monitoring (SRM)
233 transitions. Spectrum is available in Supplementary Material S2.
234
235 *Inhibition growth test.* M9 overnight cultures of *S. flexneri* tet^R were used to prepare a
236 200 µL microcosms in a 96 well-plate. Each microcosm was made with M9 salt medium
237 at a final concentration of 10^3 cell/mL of *S. flexneri* tet^R, phenanthrene 140.90 µg/L,
238 pyrene 96.05 µg/mL, benzo(a)pyrene at 30.79 µg/mL and incremental concentrations of
239 tetracycline (Fisher, Basingstoke, UK) (0, 10, 60, 110, 160, 210, 260, 310, 360 µg/mL).
240 All stock solutions of PAH's were prepared in acetonitrile and stored in the dark at 4 ºC.
241 Control microcosms contained no PAHs. Microcosms were incubated at 30 ºC for 15
242 hours. After incubation, growth was measured by reading the absorbance at optical
243 density=OD_{595} using a 96-multiplate reader (Omega Plate Reader, Aylesbury, UK). Two
244 biological and three technical replicas were performed.
245
246 *Recovery of sensitive S. flexneri* tet^S *in presence or absence of tetracycline in three*
247 *different sectors of Thames river.* Overnight inoculum in M9 medium of *S. flexneri* tet^S
248 was washed three times with 9.89 g/L phosphate buffer saline (PBS) (Fisher,
249 Basingstoke, UK) to remove residual medium before further use. Using 96-well plates,
250 200 µL of filtered water from "upstream", "London city center" and "downstream" were
251 supplemented with a final concentration of *S. flexneri* tet^S 3.4 x 10^3 CFU/mL.
252 Microcosms were also supplemented with lethal concentration of tetracycline (10
253 µg/mL). The control samples contained no tetracycline. Number of CFUs was measured
254 at time zero before starting incubation by plating aliquots on LB plates. Microcosms were
255 incubated statically for 48 hours at 30 ºC, after which, CFUs were plated on LB agar
256 plates and counted. Four biological replicas for each experiment were performed.

257

258 *Statistical analysis.* T-tests were performed by using JMP statistical software (SAS
259 Institute, Cary, NC, USA). Dot-plot distributions were produced using the software R (R
260 Core Team).

261

262

263 **RESULTS**

264

265 *Replication and persistence of sensitive and resistant Shigella flexneri in Thames river*
266 *water.*

267 Growth of tetracycline resistant and sensitive *S. flexneri* tet^R and *S. flexneri* tet^S in
268 Thames (London, UK) river water was measured by incubating the cells at 30 ºC and 37
269 ºC for up to 48 hours in Thames river water (by mixing the three sectors in equal
270 proportions). Temperatures were chosen in order to provide optimal condition for
271 proliferation. Both strains were able to significantly replicate at approximately 0.8 Log in
272 river water in 48 hours of incubation (Figure 2). Comparison of the growth of *S. flexneri*
273 tet^R and *S. flexneri* tet^S in the river water and LB did not show significant differences,
274 which demonstrates that each pair behaves equally in tested microcosm.
275 Potential altered fitness due to phage lysis of *S. flexneri* cells was measured by plating
276 Thames waters and its serial dilutions up to 10^{-9} on LB agar layers of *S. flexneri* strains.
277 No occurrence of plaques of lysis was observed in any dilutions within 1 week of
278 incubation at 30 ºC (plates were observed daily).
279 We also observed that in absence of selective pressure resistant *S. flexneri* tet^R maintain
280 the resistance genes within 7 days in microcosms. In addition we tested if extracellular
281 DNA present in river water was able to transfer tetracycline resistance cassette via

horizontal gene transfer in sensitive *S. flexneri* strains. No acquisition of tetracycline resistance cassette was observed within 48 hours of incubation.

Fitness experiments in Thames river water: Pollution downgrades the fitness of resistant bacteria.

S. flexneri tet^R and *S. flexneri* tet^S were used to measure the fitness of such a pair in Thames water. This isogenic pair is particularly interesting since the sensitive *S. flexneri* tet^S naturally lost the resistance from a clinical isolate, simulating an occurrence that could happen in nature.

In the downstream microcosms without tetracycline and sub-lethal concentration (10 ng/mL) the fitness of the two strains was equal after 48 hours of incubation (Figure 3). Interestingly when lethal concentration of tetracycline (10 µg/mL) was added to the microcosms, resistant bacteria did not significantly outcompete the sensitive strain (Figure 3). This is quite intriguing, since 10 µ/mL of tetracycline were added to the microcosm. We therefore repeated the experiments with water sampled upstream of London city center. When microcosms were repeated in this condition the resistant *S. flexneri* tet^R strain was significantly outcompeting the sensitive one as expected (Figure 4). We attributed the unexpected changes in fitness to the pollution in the downstream microcosms.

Analysis of polycyclic aromatic hydrocarbons and tetracycline degradation in Thames river water.

In order to explain why the resistant bacteria were not outcompeting the sensitive at lethal concentration of tetracycline in downstream microcosms, some of the principal pollutants, polycyclic aromatic hydrocarbons (PAHs) (Table I) and chemical degradation of tetracycline (Table II), were measured. In particular, phenanthrene, pyrene and

benzo(a)pyrene were 128, 171 and 128 times more concentrated downstream when compared with upstream respectively (Table I).

Degradation of tetracycline was also measured (Table II). It was found that up to 40% of the antibiotic had degraded within the first 3 days. However, not to such a level that could be used to explain the ability of the sensitive cells to compete with the resistant cells.

Fitness of sensitive and resistant S. flexneri in M9 medium enriched with PAHs.

Phenanthrene, pyrene and benzo(a)pyrene were about 120 times more concentrated downstream of the Thames river when compared to upstream. We tried to replicate the fitness observed in the Thames downstream microcosm by generating synthetic microcosms using M9 medium with the same concentrations of phenanthrene, pyrene and benzo(a)pyrene found in downstream river water (Table I).

When M9 microcosms were tested the sensitive *S. flexneri* tet^S was found to outcompete the resistant one in absence of tetracycline or at sub-lethal concentration (Figure 5 A, B). When tetracycline was added at lethal concentration 10 µg/mL, the resistant strains were fully outcompeting the sensitive ones (Figure 5, C). Similar results were obtained when PAHs were added to the microcosms (Figure 5 D, E and F). The PAHs at the same concentrations as found in the river were unable to simulate the fitness observed in the downstream sample. Clearly other pollutants are contributing to the effect observed in the river water.

Inhibition of S. flexneri tet^R *growth in presence of PAHs at incremental concentration of tetracycline.* While the *in vitro* fitness test performed using M9 did not show a significant effect as observed with the river samples, an appreciation of the role of PAHs in the M9 microcosms was measured in an inhibition test (Figure 6). Resistant *S. flexneri* tet^R was

grown in M9 microcosms in presence and absence of PAHs at incremental concentration of tetracycline. As the concentration of tetracycline increased, the growth in presence of PAHs was significantly reduced compared to the microcosms without PAHs.

Recollection of sensitive S. flexneri tet^S *cells in presence of tetracycline in different microcosms.*

We therefore estimated the contribution of the total chemical environment by measuring the persistence of sensitive *S. flexneri* tet^S within 48 hours of incubation in Thames river water microcosms and M9 medium as a control enriched with lethal concentration of tetracycline (Figure 7). An initial inoculum of 3.4 Log(CFU/mL) of sensitive *S. flexneri* tet^S was inoculated in the microcosms. CFUs were counted after 48 hours of incubation at 30 ºC. When no tetracycline was added an increase of ~0.8 log was measured in all the river sectors. In M9 medium the growth increased by ~2 log (Figure 7, A). Interestingly, when 10 µg/mL of tetracycline was added to the microcosms, isolation of sensitive strains seemed to be dependent on the sector, with lower isolation upstream and higher recollection downstream, following the incremental concentration of pollution. No growth was observed in M9 medium, as expected (Figure 6, B).

DISCUSSION

Understanding the fitness of resistant bacteria in an urban context is particularly significant due to the risk of ARB transfer to the population (Grill et al. 2016; Manaia 2016; Manaia et al. 2016). It has been proposed that even at extremely low abundance in environmental sources, ARB represent a high risk for human health (Manaia 2016; Manaia et al. 2016).

In this study we determined the fitness of a pair of resistant and sensitive *Shigella flexneri* strains in urban river microcosms to simulate the effect of contamination with sub-lethal (10 ng/mL) and lethal concentration (10 µg/mL) of tetracycline. Our findings showed that in the context of a polluted river the sensitive bacteria may still have an advantage even in presence of lethal selective pressure (Figure 3).

When lethal concentration of tetracycline is present in the Thames river microcosms, sensitive cells can outcompete the resistant ones in three ways: i) degradation of the antibiotic; ii) depletion of the antibiotics due to complexation; and iii) competition of the antibiotic with molecules that have similar chemical properties that are present in the environment. Interestingly, measurement of tetracycline degradation was not significant to support the first case (Table II), considering a lethal concetration of antibiotic was still in the microcosm after 48 hours. We found that degradation of tetracycline in our microcosms was similar to those reported in recent literature. Kuhne et al. (2000) demonstrated degradation of tetracycline in river water up to 20% in the first 48 hours. We therefore focused on two factors that can affect this, complexion and competition of tetracycline with pollutants in the river.

PAHs are frequently measured in polluted rivers and fluorene, phenantrene, anthracene, pyrene and benzo(a)pyrene are highly represented (Duodu et al. 2017; Tongo et al. 2017). From HPLC-fluorescence analysis of river water from the different sectors we found phenantrene, pyrene and benzo(a)pyrene, were the most abundant PAHs (Table I). Our analysis showed that the contribution of PAHs to the sensitization of the resistant strain is evident when the growth of resistant *S. flexneri* tet^R was reduced in presence of PAHs and tetracycline. Inhibition experiments showed that PAHs rendered the resistant *S. flexneri* tet^R about 20 % more sensitive to tetracycline. This could account for a

384 competition of PAHs with tetracycline rather than complexation rendering the resistant
385 strains more sensitive to the antibiotic.
386 The relationship between PAHs and antibiotic resistant genes (ARGs) has recently been
387 explored in studies on fluctuation of abundance of antibiotic resistant genes (ARGs) in
388 soil under exposure of PAHs (Sun et al. 2015). These authors showed that the
389 concentrations of pyrene and tetracycline resistance genes *tetW*, *tetM* (involved in the
390 ribosomal resistance) in soil were correlated. Dissipation of pyrene in soil was
391 associated with a significant decrease in tetracycline resistance cassettes. The
392 dissipation of the resistance genes could be explained by the activation of *tetW* and *tetM*
393 cassettes by pyrene. This mechanism comes with a high fitness cost when not under
394 selective pressure due to reduced functionality of the highly conserved center of the
395 ribosome (Wilson 2016). Therefore, we speculate that the effect of PAHs on the fitness
396 of resistant bacteria may be dual: i) Resistant strains are affected through the
397 competition of similar substrates to the same detoxification system (our findings); ii)
398 PAHs may enhance the expression of antibiotic resistant cassettes which are a burden
399 for the cell in absence of selective pressure (Sun et al. 2015). This may explain why the
400 fate of the ARGs slowly disappear from the system proposed by Sun et al. 2015, as well
401 as in our fitness tests where the resistant *Shigella* strains were more sensitive to
402 tetracycline.
403 However, these data alone do not fully explain the fitness observed in the river. In order
404 to complement these results, the sensitive *S. flexneri* tet^S was grown in presence of
405 tetracycline in river water (Figure 7). As shown in Figure 7 Panel B, the isolation of
406 sensitive cells in presence of tetracycline increases as the pollution increases. These
407 results can partially support the hypothesis that the chemicals in the environment,
408 including PAHs, may complex with tetracycline. Recent studies have shown that the
409 concentration of bioavailable PAHs has a stronger influence on the relative abundance

of tetracycline resistance gene when compared with the total concentration of tetracycline (Sun et al. 2015). The interaction with PAHs is not the only type of interaction possible. Relatively strong interactions between tetracycline and humic substances are also expected to significantly influence the reactivity, mobility and bioavailability in the environment (Gu et al. 2007). Tetracycline-resistant cells have been shown to be hypersensitive to lipophilic chelating agents and certain metal ions which may facilitate tetracycline uptake (Bochner et al. 1980; Shen et al. 2006; Zhang et al. 2014). Also, organic acids such as citric acid and oxalic acid enhance bioavailability of tetracycline in water to *Escherichia coli* for uptake and expression of antibiotic resistance (Zhang et al. 2014).

As recently stressed by Manaia and coworkers, a mere screening of antibiotic resistant genes and bacteria or antibiotic concentrations cannot be used to measure the risk for transmission to humans (Manaia 2016; Manaia et al. 2016). The risk is a function of their fitness in the environment as well as in the host, and our findings are in agreement with this view. Only limited literature is currently available, further studies must be done in order to understand to what extent these findings reflect a particular case study or if the impact is constant in other ecosystems.

A deeper analysis of the contribution of pollutants in complex environments may reveal a better understanding of bioavailability of antibiotics and fitness of antibiotic resistant bacteria. These results lead to three conclusions: i) A mere measurement of an antibiotic in a particular complex environment is only a component of the risk assessment, and does not reflect bioavailability. ii) The contribution of the PAHs in the fitness of ARB is underestimated and would require additional studies. iii) It is important to assess the contribution of complexation versus competition of PAHs with antibiotics. This contribution could also be included in mathematical model(s) for the characterization of

bacterial growth and predicting the fitness associated with drug resistance (Guo et al. 2012).

In conclusion, our findings show that in a polluted river context the sensitive *S. flexneri* cells may still have an advantage even in the presence of selective pressure and fitness tests provide an additional tool to measure bioavailability.

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573
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575 **CAPTIONS**
576
577 **Table I.** Concentrations of selected PAHs in different sampling sites in Thames river.
578
579 **Table II.** Degradation of tetracycline in different sectors of the Thames river.
580
581 **Figure 1. Sampling points and river flow of Thames river, London, UK.** The map
582 represents the Thames river, London, UK. Dotted arrow on the top right represents the
583 water flow. Black arrows show sampling points.
584
585 **Figure 2. Proliferation and persistence of *S. flexneri* tet^R and *S. flexneri* tet^S in**
586 **Thames river water.** Panel A and B, growth of *S. flexneri* tet^R and *S. flexneri* tet^S at 37

587 ºC. Panel C and D growth of *S. flexneri* tet^R and *S. flexneri* tet^S at 30 ºC. The box-plots
588 show the lower and upper quartiles (determined by the boxes), thick lines within the box
589 are the median values, and the whiskers indicate the degree of dispersion of the data.
590 Asterisks represent significant difference in proliferation (t-test).
591
592 **Figure 3. Fitness of isogenic pair of *S. flexneri* tet^R and *S. flexneri* tet^S in Thames**
593 **river water "downstream".** Panel A shows the fitness of *S. flexneri* in downstream river
594 water without any tetracycline. In panel B the concentration is sub lethal (10 ng/mL), in
595 Panel C the concentration is lethal (10 µg/mL). In the Log(CI) where the CI is 0 there is
596 not fitness cost. When the fitness is negative the sensitive bacteria are outcompeting the
597 resistant. When the CI is positive the resistant are outcompeting the sensitive. The box-
598 plots show the lower and upper quartiles (determined by the boxes), thick lines within the
599 box are the median values, and the whiskers indicate the degree of dispersion of the
600 data.
601
602 **Figure 4. Fitness of isogenic pair *S. flexneri* tet^R and *S. flexneri* tet^S in Thames**
603 **water in "upstream" microcosm in presence of lethal concentration of tetracycline.**
604 The interpretation of the Log(CI) is reported in Figure 3. The box-plots show the lower
605 and upper quartiles (determined by the boxes), thick lines within the box are the median
606 values, and the whiskers indicate the degree of dispersion of the data.
607
608 **Figure 5. Fitness of isogenic pair *S. flexneri* tetR and *S. flexneri* tet^S in M9 salt**
609 **medium in presence and absence of selected PAHs.** Panels A, B and C show the
610 fitness of the *S. flexneri* pair in M9 only at different concentrations on tetracycline. Panels
611 D, E and F includes PAHs (Table I and Materials and Methods). The interpretation of the
612 Log(CI) is reported in Figure 3. Asterisks represent significant differences. Lines in the

613 panels are indeed box plots. All replicas were behaving the same, shrinking the boxes
614 into lines. The box-plots show the lower and upper quartiles (determined by the boxes),
615 thick lines within the box are the median values.
616
617 **Figure 6. Reduction of growth of *S. flexneri* tet^{R} in presence of PAHs at different**
618 **concentrations of tetracycline.** PAHs concentration was equal to the concentration
619 measured in "downstream" river (Table I). Error bars represent standard error.
620
621 **Figure 7. Recovery of *S. flexneri* tet^{S} in presence or absence of tetracycline in**
622 **different sections of the Thames river.** Panel A. *S. flexneri* tet^{S} inoculated in different
623 sampling sites of the Thames (upstream, city center, downstream and M9 as a control).
624 Panel B. *S. flexneri* tet^{S} inoculated in the same microcosms with addition of lethal
625 tetracycline (10µg/mL). Error bars represent standard error.
626
627 **Supplemental Figure S1.** Sampling site and CSOs location and diagram with the
628 experiments.
629
630 **Supplemental Figures S2.** Fluorescent detector excitation and emission wavelengths
631 for analysis of PAH's, Pulse periods was 0.2 s and pulse width 1 s. Spectra for PAHs
632 and tetracycline analysis.
633
634