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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.

1. 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 [1,2]. 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 [3,4]. 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 1.6×10^2 ng/g [5–7]. 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 [8–10]. In addition, the use of disinfectants in urban wastewater treatment plants can promote a residual microbial community that is more resistant to antibiotics [11]. 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 [12].

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 [13–16]. 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 [17,18]. 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 [19], nor fluoroquinolones resistance in *Salmonella enterica* serovar Typhi have been shown not to have a disadvantage over the sensitive parent strain

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[20]. 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 [21]. 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 [22]. *S. flexneri* 2a strain naturally carries multi antibiotics resistance and it can infect at very low infection dose (tens of cells) [23]. In recent years, multidrug resistance (MDR) in several *Shigella* strains has become a public health problem [24,25]. Genes harbored within the *Shigella* resistance locus pathogenicity island (SRL PAI) were identified as contributors to the resistance phenotype [26]. 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) [26]. Currently no studies have been reported that show a relationship between the presence of *S. flexneri* SRL island with survival of the bacteria in the environment and its response to the antibiotics whilst reproducing in such environment.

Our findings show that in an *in vitro* polluted river context (microcosms) the sensitive *S. flexneri* cells may still have an advantage in presence of lethal concentration of antibiotics and the competitive test provides a useful indication in terms of bioavailability of the antibiotic tetracycline.

2. Materials and methods

2.1. Sampling sites

Thames river (London, UK) sampling sites were chosen in three different sectors according to the river flow: upstream of the city center, city center, and downstream of the city center (Fig. 1). Each sampling sector was approximately 20 km apart from each other. For each sector, three 2 L samples were taken in different parts of the river on September 2016 (Fig. 1 and Supplementary materials S1). These were taken from the surface of the river using polyethylene terephthalate bottles and frozen within 7 h of sampling. All samples were transferred to the laboratory within 2 weeks for the generation of microcosms.

Samples from city center and downstream sectors were exposed to combined sewer overflows (CSOs). CSOs release wastewater in the Thames when the water flow is intense, eventually contaminating the river with untreated wastewater discharges [27,28].

2.2. Strains used in this study

Strains used in the competition analysis were the resistant *Shigella flexneri* 2a YSH6000 [29] (labeled as *S. flexneri* tet^R) and sensitive *S. flexneri* 2a 1363 (labeled as *S. flexneri* tet^S), with a spontaneous deletion of the SRL island [30]. Strains were cultured overnight in LB medium (Oxoid, Basingstoke, UK), or 1X Minimal Salt (M9 medium) (Invitrogen, Carlsbad, US). M9 medium was prepared according to manufacturer's specifications with 12.5 μM nicotinic acid (Sigma-Aldrich St. Louis, MS, USA) (*S. flexneri* tet^R and *S. flexneri* tet^S are auxotroph for nicotinic acid) and 0.2% w/v of glucose (Sigma-Aldrich St. Louis, MS, USA) were used to generate the M9 final medium.

2.3. Water filtration

200 mL from each river sampling site were filtered twice using Whatman paper No 1 (particle retention 11 μm) (Sigma-Aldrich St. Louis, MS, USA) then filtered twice using 0.22 μm filters (Billerica, MA, USA) to ensure the removal of the microbial community. The three samples from the same river sector were combined to form "upstream", "center" and "downstream" samples. These were subsequently aliquoted into 50 mL Falcon tubes (Fisher, Basingstoke, UK) and frozen at −20 °C until analysis.

2.4. Test for loss or acquisition of tetracycline cassette in S. flexneri strains

We tested if the tetracycline resistance carried by *S. flexneri* tet^R was persistent within a 1-week period. Microcosms without selective pressure were prepared: i) A sample containing a mixture of water from the three sectors of Thames and a control containing 0.85% wt/v saline solution were prepared. Microcosms were inoculated separately with 10^5 cells/mL of the resistant bacterial strain and incubated at 30 °C for 0, 2, 5 and 7 days. Following incubation 100 CFU were picked and patched onto LB selective medium containing tetracycline 10 μg/mL.

To test horizontal tetracycline cassette acquisition, 10^5 cells/mL of

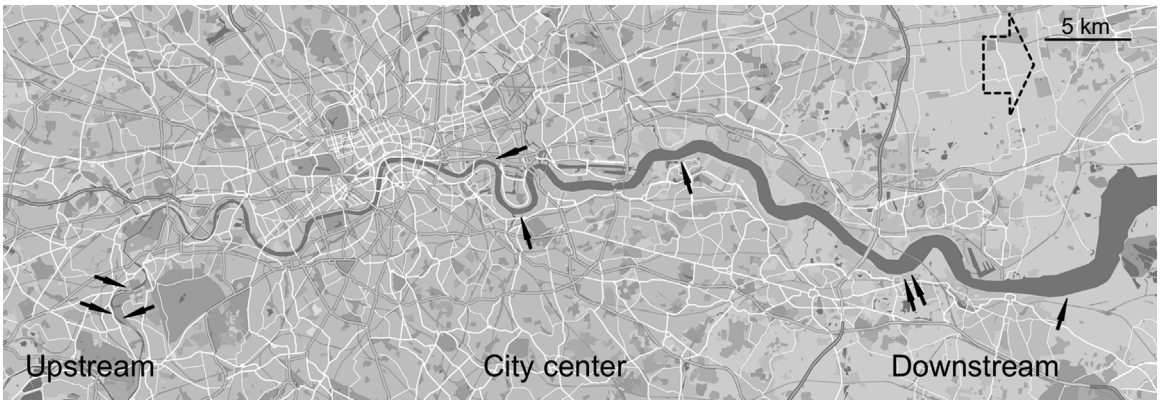

Fig. 1. Sampling points and river flow of Thames river, London, UK. The map represents the Thames river, London, UK. Dotted arrow on the top right represents the water flow. Black arrows show sampling points.