A method to assess bioavailability of antibiotics in anthropogenic polluted ecosystems by using a bacterial fitness test

Massimiliano Marvasi [1], Alessandro Canali [1], Brunella Perito [1], Ajit Shah [2], Vlad Serafim [2]*

[1] University of Florence, Department of Biology, Florence, Italy.

[2] Middlesex University London, Department of Natural Sciences, London, UK

* Corresponding Author: Massimiliano Marvasi, University of Florence, Via Madonna del Piano 6. Sesto Fiorentino, Florence, Italy. Tel. 0039 0554574749. Email: massimiliano.marvasi@unifi.it

Abstract

Antibiotics released in the environment exert a selective pressure on the resident microbiota. It is well accepted that the mere measurement of antibiotics does not reflect the actual bioavailability. In fact, antibiotics can be adsorbed or complexed to particles and/or chemicals in water and soil. Bioavailable concentrations of antibiotics in soil and water are subjected to great uncertainty, therefore biological assays are increasingly recognized as that allow an indirect determination of the residual antibiotic activity. Here we propose how a fitness test for bacteria can be used to qualitatively assess the bioavailability of a specific antibiotic in the environment. The findings show that by using a pair of resistant and sensitive bacterial strains, the resulting fitness can indirectly reflect antibiotic bioavailability. Hence, this test can be used as a complementary assay to other biological and chemical tests to assess bioavailability of antibiotics.

Keywords: antibiotics, river water, tetracycline, fitness test, bioavailability, ecosystems

Introduction

Antibiotics are frequently found in river waters and soil, near farms where they are used in prophylaxis and production of livestock [1, 2]. Hospitals in developed and developing countries are also subjected to accidental release of antibiotics that can be detected in the environment [3]. It is generally accepted that residual antibiotics support selective pressure on resident microbiota. Residual antibiotics may support the spread of antibiotic resistance genes (ARG) or bacteria (ARB) through the microbial communities [4, 5].

While efficient extraction methods combined with high sensitivity analyses (i.e. liquid chromatography/mass spectrometry) can provide accurate quantification of antibiotics and their transformation products, the measurements do not necessarily reflect bioavailable fractions [6]. Recent studies have shown that even if antibiotics are detected in soil (and in some cases are abundant), they are not necessarily bioavailable due to interaction with soil particles or other chemicals [7-10]. If the environmental conditions change, the bound antibiotic may be released in the environment and this will redefine the environmental selective pressure. For example, reactivity, mobility and bioavailability of tetracycline is affected by humic substances in river water [8]. In other cases heavy metals and organic acids such as citric acid and oxalic acid have been shown to enhance bioavailability of tetracycline in water to *Escherichia coli* [11-13].

The selective pressure can be indirectly measured through screenings of abundance of ARG and ARB. However, such assessment is not free from errors: the abundance may result from wildlife translocation or horizontal gene transfer [14].

Complex techniques are available to assess bioavailability of antibiotics, such as gene reporters or expression of specific genes analysed by qPCR or chemical analyses [8, 13, 15]. Here we report a method to semi-quantitatively assess bioavailability in river water by using a pair of isogenic bacteria in a fitness test. The rationale that supports this method, is that the fitness of an isogenic pairs (one resistant, one sensitive) changes in accordance with the

selective pressure of a specific environment. Therefore, comparison of the fitness of different microcosms would not only reflect changes of bioavailability but also degradation of the antibiotic itself.

This method was tested on two different anthropogenic polluted rivers: The river Thames (London, UK) and Arno river (Florence, Italy). The method was tested with two different types of bacteria, *Shigella flexneri* and *Escherichia coli*. This method could be used to support risk assessment.

Material and Methods

Sampling sites. river Thames (London, UK) sampling site was chosen downstream of the city centre and coordinates of sampling sites are available in [7]. Arno river (Florence, Italy) water was sampled downstream of the city centre (the coordinates of the sampling points are: lat 43.772935; lon 11.241877, and Supplemental Material 1). For each sector, three 2 L samples were taken on the same day from the surface of the rivers 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 [7].

Samples from the 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 [16, 17].

Strains used in this study. Strains used in the fitness test were the tetracycline resistant *Shigella flexneri* 2a YSH6000 [18] (labelled as *S. flexneri* tet^R) and the tetracycline sensitive *S. flexneri* 2a 1363 (labelled as *S. flexneri* tet^S) with a spontaneous deletion of the *Shigella* Resistance Locus (SRL) island [19]. 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. The *Escherichia coli* strains used were: the tetracycline resistant *E. coli* XL-1 tet^R (genotype: *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F´ *proAB lacI*qZ∆*M15* Tn*10*]) (where Tn10 carries the *tetRA* cassette) and the tetracycline sensitive *Escherichia coli* DH5a tet^S as sensitive strain [20].

Water filtration. Three (200 mL) aliquots of water from each sampling site were filtered twice using Whatman paper No 1 (particle retention 11 µm) (Sigma-Aldrich St. Louis, MS, USA) and then filtered twice using 0.22 µm filters (Billerica, MA, USA) to ensure all microbes were removed. The three samples from the same river sector were combined. These were subsequently aliquoted into 50 mL Falcon tubes (Fisher, Basingstoke, UK) and frozen at -20 ºC until analysis.

Test for loss of tetracycline cassette in S. flexneri and E. coli strains. We tested if the tetracycline resistance cassette carried by *S. flexneri* tet^R and *E. coli* XL-1 tet^R was persistent within 48 h in microcosms without selective pressure. Thames or Arno water and a control containing 0.85 % w/v NaCl solution were prepared. Microcosms were inoculated separately with 10^5 cells/mL of the resistant strains and incubated at 30 ºC for up to 48 h. Following incubation, cells were recovered in LB without selective pressure and 100 CFU were picked and patched onto LB selective medium containing tetracycline 10 µg/mL (Sigma-Aldrich St. Louis, MS, USA). The number of colonies that had lost resistance were counted. Three replicas were performed.

To test *acquisition of tetracycline cassette* from the river water (in the unlikely event of phages carrying a *tetAR* cassette), 10^5 cells/mL of the sensitive strains were incubated in Thames water or Arno water. Cells were incubated for 48 h at 30 ºC. After incubation 25 µL aliquots were plated onto LB plate supplemented with tetracycline at 10 µg/mL for the detection of resistant colonies. Three replicas were performed.

Phage lysis test. 500 µL of overnight LB culture of resistant and sensitive *S. flexneri* and *E. coli* were resuspended in 20 mL of molten LB agar. Once plates had solidified, 0.5 mL of river water sample, ranging from undiluted to 10^{-9} with water, were plated onto the LB plate. Plates were incubated at 30 ºC for up to one week. Plates were observed daily for up to one week to identify plaques of lysis. Three replicas were performed.

Fitness test. Overnight M9 cultures of resistant and sensitive *S. flexneri* or *E. coli* were washed three times with sterile 9.89 g/L phosphate buffer saline (PBS) (Fisher, Basingstoke, UK) to remove the residual medium before inoculation. Washed resistant and sensitive cells were mixed in a 1:1 ratio by using equal value of absorbance at OD_{595} using a UV/VIS spectrophotometer (Helios Epsilon, Thermo Scientific, Waltham, MA, USA). To confirm that the 1:1 ratio was achieved, a sample of 200 colonies of the 1:1 mixture was immediately screened on LB selective medium (10 µg/mL tetracycline). River water microcosms were enriched with tetracycline at concentration of 10 ng/mL (sub-lethal) and 10 µg/mL (lethal) and no tetracycline as control. 10^5 CFU/mL of the 1:1 mixture were inoculated into the river water and an aliquot was used as "time 0" (R_{in}/S_{in} in the equation below). Microcosms were then incubated at 30 °C for 48 h in sterile tubes which were kept static to simulate stagnant water. The tubes were opened daily under the BL2 cabinet for 10 min to allow gas exchanges and they were briefly shaken. All aliquots were plated onto 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 exact number of replicas are reported in each Figure's caption.

Measurement of tetracycline. The methods is the same used in [7]. Briefly, an X-LC UHPLC system (JASCO, UK) coupled to an API 3000 triple quadrupole mass spectrometer (Applied Biosystems, Warrington, UK) was used. The chromatographic separation was achieved using an Ascentis Express C18 column (Sigma Aldrich, Poole, UK). A binary gradient of A - 0.1% formic acid in water and B - 0.1 % formic acid in acetonitrile was used. The elution profile used started at 10% B then increased at 75% over 5 min and maintained at this level for 1 min and then returned to 10% B for 3 min to equilibrate the column. The flow rate was set at 0.21 mL/min. A volume of 10 µL was injected per run and the column oven temperature was set at 50˚C. The MS electrospray source was operated in the positive-ion mode. The MRM transitions for tetracycline *m/z* 445.3 → 410.1 and 445.3 → 154.5 were monitored simultaneous. The detection limit (LOD) was established as the lowest concentration of the calibration standard that was detected with a signal-to-noise (S/N) ratio ≥ 3:1 while the quantification limit (LOQ) was established as the lowest concentration of the calibration standard that was detected with a signal-to-noise (S/N) ratio ≥ 10:1. LOD and LOQ were 2 and 10 ng/mL respectively. The retention time was 3.1 min. Tetracycline was identified by retention times (Rt) and by 2 selected reaction monitoring (SRM) transitions. Measurements were made by using two replicas for each condition and two injections for each replica.

Statistical analyses: ANOVA and Tukey separations were performed with JMP(SAS) statistical software. In all analyses a cut-off of $p<0.05$ was used. Graphs were done with Prism 8.0.

Results and Discussion

168 Preliminary experiments were performed to assure: i) that the resistance cassette was not lost
169 from the resistant strain; ii) that there was no acquisition of tetracycline resistance from the
170 environment; and iii) to check for lysis of plaque due to possible presence of phage. It is
171 important to implement these controls before running the fitness test. In fact, presence of
172 phage or loss of the resistance cassette would affect the fitness test. For example, it is
173 important to be sure that recovery of sensitive bacteria is due to selective pressure and not
174 due to phage lysis. Once these variables were excluded, the fitness tests were performed.
175 *Shigella flexneri* was first tested in a minimum M9 medium (Figure 1) as control experiment.
176 At time 0 the 1:1 mixture of *S. flexneri* 2a YSH6000 and *S. flexneri* 2a 1363 were inoculated.
177 After 48 h of incubation the sensitive strain was fully out-competing the resistant one in
178 absence of tetracycline or at sub-lethal concertation of tetracycline (10ng/mL) (Figure 1).
179 Fitness of the pair *E. coli* tet^R/tet^S showed the same results (data not shown). This was
180 expected, the resistant strains harbour the complex efflux machine (*tetRA* genes), which slows
181 replication in absence of selective pressure. Other studies have also confirmed the metabolic
182 burden of the *tetRA* cassette in absence of selective pressure in *E. coli* [21, 22]. In contrast,
183 in the of presence of lethal tetracycline (10µg/mL) the resistant strain was out-competing the
184 sensitive one (Figure 1).
185 *Shigella flexneri* isogenic pair was then tested in two different environments: Arno river water
186 (Florence, Italy) and river Thames water (London, UK) (Figure 2 A and B, respectively). The
187 experiment performed using Arno river water showed that sensitive cells were more fit in
188 absence of selective pressure. Resistant *Shigella* was out-competing the sensitive cells in
189 presence of lethal concentration of tetracycline (10µg/mL) (Figure 2 A). Using Arno water we
190 observed similar results to those of the control (Figure 1). In contrast, in the polluted river
191 Thames water, sensitive *Shigella* strain was not out-competed by the resistant strain in
192 presence of lethal concentration of tetracycline (Figure 2 B). The highest level of degradation
193 that was observed was around 40% and this was not enough to deplete lethal selective
194 pressure (Table 1). Therefore, variations of fitness in Figure 2 is an example of the contribution

195 of the chemical environment, showing the tetracycline was still present but not bioavailable in
196 river Thames water.
197 Finally, fitness of *E. coli* was measured in Arno river water (Figure 3). After 48h of incubation
198 the resistant strain was out-competing the sensitive at lethal concentration (10 µg/mL), as
199 expected. In Arno water we can conclude that the chemical environment of the water was not
200 affecting bioavailability of tetracycline. This can be ascribed to a number of different factors:
201 anthropogenic pollution, natural presence of colloidal substances (which chelate antibiotics),
202 heavy metals [6, 23]. It may be difficult to study absorption of tetracycline. Recent papers have
203 reported surface-bridging mechanism between calcium salts, clay and tetracycline at alkaline
204 pHs. Similar studies should be done by using river water [23]. Ionic strength is involved in
205 sorption of tetracycline on different clay particles [23]. As mentioned by Aga and co-workers
206 (2016) there is a need to complement chemical analysis with biological assays that can
207 provide information on bioavailability [6]. Our manuscript shows a biological assay that could
208 be used for this purpose. It would be appropriate to see more studies linking the fitness tests
209 proposed in this manuscript with multiresidue methods, designed to measure sub-ng
210 concentrations in complex mixtures [6].
211 A number of fitness tests have been reported in the literature that can be used to achieve the
212 same semi-quantitative assessment, some involving studies on communities [24], other
213 studying relative abundance of bacteria in urban wastewater treatment plants [25]. A different
214 approach was used by Chait and collaborators [26]: in their study they used YFP- labelled,
215 tetracycline-sensitive (green) and CFP-labelled, tetracycline-resistant (red) *E. coli* that were
216 mixed and grown together over a diffusing gradient in agar containing tetracycline [26].
217 Heterogeneous conditions were found to influence the selective advantage or disadvantage
218 of antibiotic resistance, resulting in detection of prevalence of green or red fluorescence on
219 the plate [26].
220 Another type of bioassay uses a *E. coli* MC4100/pTGM bioreporter strain. In this system, the
221 deactivation of the TetR repressor protein in the $P_{tet(A)}$ promoter, activates *gfp* gene
222 transcription. Tetracycline that enters the cell was measured using fluorescence detection.

The level of emission was found to be directly related to the amount of tetracycline that accumulated inside *E. coli* cells [27].

Although advances in LC/MS/MS instrumentations have facilitated the detection of trace level of antibiotics, assays for the direct effect of bioavailability are important tools to better understand the impact of antibiotics in agroecosystems and accurately predict their contribution to the development of antibiotic resistance in bacteria [6]. Fitness test can provide additional tools for risk assessment. An example: the use of fitness tests during mock release of antibiotics in a specific environment would allow the study of the fate in terms of persistence and replication of dangerous resistant pathogenic bacteria [7].

Captions

Figure 1. Fitness of the pair *S. flexneri* tet^R/tet^S exposed to different concentrations of tetracycline in M9 salt medium. When the Log(CI) is 0 there is not fitness cost, both strains replicate at the same speed. When the Log(CI) is negative the sensitive bacteria are out-competing the resistant ones. When the Log(CI) is positive the resistant bacteria are out-competing the sensitive ones. Error bars represent the standard error. At time 48h error bars were absent because all samples behaved the same. Different letters represent different means. Fitness of the pair *E. coli* tet^R/tet^S showed the same results (data not shown). Two biological replicas were performed (50 colonies were screened in each replica for a total of 100 colonies screened using each condition).

Figure 2. Fitness of the pair of *S. flexneri* tet^R/ tet^S in Arno river and river Thames water. The interpretation of the Log(CI) is reported in caption of Figure 1. Panel A shows the fitness of *S. flexneri* tet^R/ tet^S in Arno river water at different concentrations of tetracycline. Panel B shows the fitness of *S. flexneri* tet^R/ tet^S in river Thames downstream river water. Error bars represent the standard error. Different letters represent different means.

Two biological replicas were performed (300 colonies were screened in each replica for a total of 600 colonies screened in each condition).

Figure 3 Fitness of the pair of *E. coli* tet^R/ tet^S in Arno river water. The interpretation of the Log(CI) is reported in caption of Figure 1. The fitness of *E. coli* tet^R/ tet^S in Arno river water is shown at different concentrations of tetracycline. Error bars represent the standard error. Different letters represent different means. Three biological replicas were performed (100 colonies were screened in each replica for a total of 300 colonies screened in each condition).

Supplemental Material 1: Arno river (Florence, Italy) sampling site. Detailed sampling sites for river Thames (London, UK) are available at [7].

REFERENCES

1. Boxall, A. *Identifying Hotspots of Resistance Selection from Antibiotic Exposure in Urban Environments around the World*. in *Europe 29 th Annual Meeting* 2019. Helsinki: SETAC.
2. Brown, P.C., et al., *Impact of the particulate matter from wastewater discharge on the abundance of antibiotic resistance genes and facultative pathogenic bacteria in downstream river sediments.* Science of The Total Environment, 2019. **649**: p. 1171-1178.
3. Gros, M., S. Rodríguez-Mozaz, and D. Barceló, *Rapid analysis of multiclass antibiotic residues and some of their metabolites in hospital, urban wastewater and river water by ultra-high-performance liquid chromatography coupled to quadrupole-linear ion trap tandem mass spectrometry.* Journal of Chromatography A, 2013. **1292**: p. 173-188.
4. Knapp, C.W., et al., *Evidence of increasing antibiotic resistance gene abundances in archived soils since 1940.* Environment Science and Technology, 2010. **44**: p. 580-587.

5. Allen, H.K., et al., *Treatment, promotion, commotion: antibiotic alternatives in food-producing animals.* Trends in microbiology, 2013. **21**(3): p. 114-119.
6. Aga, D.S., et al., *Challenges in the Measurement of Antibiotics and in Evaluating Their Impacts in Agroecosystems: A Critical Review.* Journal of Environmental Quality, 2016. **45**(2): p. 407-419.
7. Maruzani, R., et al., *Effect of anthropogenic pollution on the fitness of tetracycline sensitive Shigella flexneri in Thames river water*. 2018. Journal of Environmental anche Chemical Engineering **6**(1): p. 19-27.
8. Gu, C., et al., *Complexation of the antibiotic tetracycline with humic acid*. 2007. Chemosphere **66**(8): p. 1494-1501.
9. Wang, H., et al., *Transformation of Tetracycline Antibiotics and Fe(II) and Fe(III) Species Induced by Their Complexation.* Environ Sci Technol, 2016. **50**(1): p. 145-53.
10. Menz, J., et al., *Bioavailability of Antibiotics at Soil–Water Interfaces: A Comparison of Measured Activities and Equilibrium Partitioning Estimates.* Environmental Science & Technology, 2018. **52**(11): p. 6555-6564.
11. Bochner, B.R., et al., *Positive selection for loss of tetracycline resistance.* Journal of Bacteriology, 1980. **143**(2): p. 926-933.
12. Shen, G., Y. Lu, and J. Hong, *Combined effect of heavy metals and polycyclic aromatic hydrocarbons on urease activity in soil*. 2006. Ecotoxicology and Environmental Safety **63**(3): p. 474-480.
13. Zhang, Y., et al., *Organic acids enhance bioavailability of tetracycline in water to Escherichia coli for uptake and expression of antibiotic resistance*. 2014. Water Research **15**: p. 98-106.
14. Manaia, C.M., *Assessing the Risk of Antibiotic Resistance Transmission from the Environment to Humans: Non-Direct Proportionality between Abundance and Risk.* Trends in microbiology, 2017. **25**(3): p. 173-181.
15. Moller, T.S.B., et al., *Relation between tetR and tetA expression in tetracycline resistant Escherichia coli.* BMC Microbiology, 2016. **16**(1): p. 39.

16. Group, S., *Thames Tideway Strategic Study. Doc Ref: 8.1.2 Steering Group Report*. 2005.
17. Schreiber, C., et al., *Two decades of system-based hygienic–microbiological research in Swist river catchment (Germany).* Environmental Earth Sciences, 2016. **75**(21).
18. Rajakumar, K., C. Sasakawa, and B. Adler, *A spontaneous 99-kb chromosomal deletion results in multi-antibiotic susceptibility and an attenuation of contact haemolysis in Shigella flexneri 2a.* Journal of Medical Microbiology, 1996. **45**(1): p. 64-75.
19. Luck, S.N., et al., *Excision of the Shigella Resistance Locus Pathogenicity Island in Shigella flexneri Is Stimulated by a Member of a New Subgroup of Recombination Directionality Factors.* Journal of Bacteriology, 2004. **186**(16): p. 5551-5554.
20. Sambrook, J., *Molecular cloning : a laboratory manual*. 2001: Third edition. Cold Spring Harbor, N.Y. : Cold Spring Harbor Laboratory Press, [2001] ©2001.
21. Linkevicius, M., L. Sandegren, and D.I. Andersson, *Mechanisms and fitness costs of tigecycline resistance in escherichia coli.* Journal of Antimicrobial Chemotherapy, 2013. **68**(12): p. 2809-2819.
22. Johnson, T.J., et al., *In Vivo transmission of an incA/C plasmid in Escherichia coli depends on tetracycline concentration, and acquisition of the plasmid results in a variable cost of fitness.* Applied and Environmental Microbiology, 2015. **81**(10): p. 3561-3570.
23. Figueroa, R.A., A. Leonard, and A. MacKay, *Modeling tetracycline antibiotic sorption to clays*. Environmental Science and Technology 2004. **38**(2): p 476-483.
24. Ternent, L., et al., *Bacterial fitness shapes the population dynamics of antibiotic-resistant and -susceptible bacteria in a model of combined antibiotic and anti-virulence treatment.* Journal of theoretical biology, 2015. **372**: p. 1-11.
25. DiCesare, A., et al., *Fitness and Recovery of Bacterial Communities and Antibiotic Resistance Genes in Urban Wastewaters Exposed to Classical Disinfection Treatments.* Environmental Science and Technology, 2016. **50**(18): p. 10153-10161.

26. Chait, R., et al., *Pervasive selection for and against antibiotic resistance in inhomogeneous multistress environments.* Nature Communications, 2016. **7**: p. 10333.
27. Zhang, Y., et al., *Bioavailability of tetracycline to antibiotic resistant Escherichia coli in water-clay systems.* Environ Pollut, 2018. **243**(Pt B): p. 1078-1086.