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Minimal selective concentrations of tetracycline in complex aquatic bacterial biofilms

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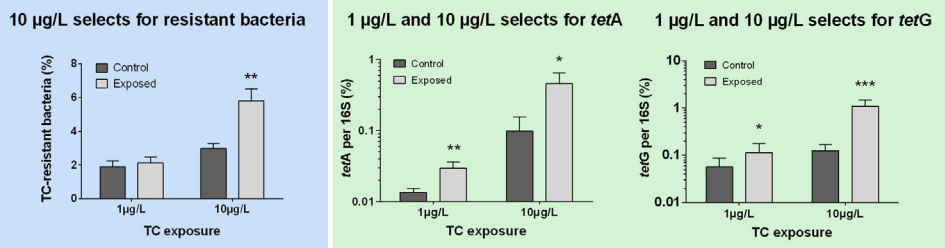
HIGHLIGHTS

- Methods to determine minimal selective concentrations of antibiotics were compared.
- One μg/L tetracycline selects for *tet*A and *tet*G genes in freshwater biofilms.
- Ten μg/L tetracycline selects for phenotypic resistance.
- Selective concentrations overlap with those reported in sewage treatment plants.
- Approach described could be used in risk assessment of other antibiotics as well.

GRAPHICAL ABSTRACT



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ABSTRACT

Selection pressure generated by antibiotics released into the environment could enrich for antibiotic resistance genes and antibiotic resistant bacteria, thereby increasing the risk for transmission to humans and animals. Tetracyclines comprise an antibiotic class of great importance to both human and animal health. Accordingly, residues of tetracycline are commonly detected in aquatic environments. To assess if tetracycline pollution in aquatic environments promotes development of resistance, we determined minimal selective concentrations (MSCs) in biofilms of complex aquatic bacterial communities using both phenotypic and genotypic assays. Tetracycline significantly increased the relative abundance of resistant bacteria at 10 μg/L, while specific *tet* genes (*tet*A and *tet*G) increased significantly at the lowest concentration tested (1 μg/L). Taxonomic composition of the biofilm communities was altered with increasing tetracycline concentrations. Metagenomic analysis revealed a concurrent increase of several *tet* genes and a range of other genes providing resistance to different classes of antibiotics (*e.g. cml*A, *flo*R, *sul*1, and *mph*A), indicating potential for co-selection. Consequently, MSCs for the *tet* genes of ≤1 μg/L suggests that current exposure levels in *e.g.* sewage treatment plants could be sufficient to promote resistance. The methodology used here to assess MSCs could be applied in risk assessment of other antibiotics as well.

Abbreviations: ARG, antibiotic resistance gene; CFU, colony forming unit; EC50, concentration at 50% effect; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MIC, minimal inhibitory concentration; MSC, minimal selective concentration; PICT, pollution-induced community tolerance; qPCR, quantitative polymerase chain reaction; TC, tetracycline.

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1. Introduction

Antibiotic resistance genes (ARGs) have been present in the environment long before humans started using antibiotics to treat bacterial infections (D'Costa et al., 2011). However, after introduction of antibiotics as clinical agents, ARGs have become very common in human pathogens. External environments host a vast diversity of bacteria, much larger than that found in and on our bodies. Thus, the environmental resistome most likely contains many rare or undiscovered ARGs, that under a sufficient selection pressure from antibiotics could be enriched, mobilized, and end up in pathogens, which most likely have happened with many ARGs in the past (Davies and Davies, 2010).

Widespread use and misuse of antibiotics over the past 70 years has been accompanied by unintentional environmental pollution of antibiotics. Thus, there is a potential for antibiotics to cause selection pressures in humans and animals undergoing therapy, but also in every environment impacted by our waste. Measured concentrations of antibiotics in aquatic environments range from ng to μg/L as a result of excretion from humans and animals (Watkinson et al., 2009; Segura et al., 2009; Rizzo et al., 2013) while levels as high as mg/L have been detected as a consequence of direct pollution from drug manufacturing (Larsson, 2014). Determining the concentrations of antibiotics that are selective for resistance is crucial for identifying environments where increase of ARGs are predicted to occur and measures need to be taken to minimize such an increase (Ashbolt et al., 2013; Pruden et al., 2013; Brandt et al., 2015).

Tetracycline (TC) antibiotics are one of the most commonly used antibiotic classes, primarily due to their broad activity spectrum and low cost. Consumption of TCs by animals and humans in the European Union is estimated at thousands of tons each year (Kools et al., 2008; Coenen et al., 2011). Since up to 75% of consumed TCs are excreted in their active forms (Elmund et al., 1971; Agwuh and MacGowan, 2006), large amounts of TCs reach aquatic environments.

Two previous studies have reported the minimal selective concentration (MSC) of TC using two competing strains, which differ only in two aspects. They contain different marker gene(s) and the resistant strain has a transposon (Tn10), containing *tet*A. Liu et al. (2011) reported a MSC for TC of 62.5 μg/L, and later that year Gullberg et al. (2011) reported a somewhat lower MSC for TC of 15 μg/L. Discrepancies between these two MSCs may be due to differences in test strains, fitness cost, and/or sensitivity of the detection system. These studies demonstrate the ability of TC to select for a specific resistance factor in a highly simplified competition situation. Environments polluted with TCs, such as surface waters, sediments, and sewage treatment plants, contain much more complex communities with a large diversity of bacterial species and ARGs. In such communities there are ample possibilities for a range of species and strains to fill the ecological niches made available through an added selection pressure. Hence, it is not straightforward to extrapolate the selective potential of an antibiotic to entire communities based on competition experiments with only two strains. Effects of TC on microbial communities have been reported in one study where five stream mesocosms were exposed to different TC concentrations (0, 0.5, 1, 10, 100 μg/L) (Quinlan et al., 2011). That study focused on ecotoxicological effects of TC exposure on *e.g.* bacterial productivity, abundance of algae, and organic biomass. Additionally, this study reported a significant increase of phenotypic TC resistance at 0.5 μg/L. However, TC resistance increase did not follow a traditional exposure-response pattern, as exposure to 0.5 μg/L caused significantly increased TC resistance, whereas 1, 10, and 100 μg/L counterintuitively did not. Therefore, there is a need to clarify the TC exposure-response relationship in complex aquatic communities with regard to selection of TC resistance.

It is not obvious whether a phenotypic, genotypic, or taxonomic endpoint should be used when assessing the selective ability of an antibiotic in a complex microbial community. In the case of infection by a pathogen, phenotypic resistance is crucial for the treatment outcome. Hence, the ability to select for bacteria tolerating an antibiotic concentration exceeding the clinical breakpoint for a given antibiotic is a reasonable endpoint. However, in an environmental community, treatability is of less concern than *e.g.* risk for selection of mobilizable genes. Thus, genotypic resistance could be equally (or even more) relevant to study. It is of particular concern that novel ARGs could become enriched, mobilized, and transferred to pathogens (Bengtsson-Palme and Larsson, 2015). Detecting such novel ARGs is difficult, but it is reasonable to assume that they are likely to be overrepresented in more tolerant strains. Taxonomic shifts in a bacterial community, following addition of an antibiotic, thus demonstrate that a selection pressure has been exerted on the community and that sensitive strains have been replaced by more tolerant ones (Blanck, 2002). Hence, a taxonomic change is a reflection of a selection pressure that over time could contribute to the development of resistance of clinical concern.

The primary aim of this study was to determine MSCs of TC on a genotypic and phenotypic level in complex aquatic bacterial biofilms. We were also interested in identifying changes in taxonomic composition related to TC exposure. Three experiments were performed. In the first experiment, an exposure-response was established using a 10-fold dilution series of TC in order to provide a concentration range in which selection was likely to occur. This experiment also provided an initial evaluation of different endpoints, including taxonomic changes, based on their apparent sensitivity, exposure-response, and experimental/analytical throughput. In the second and third experiments, MSCs were established for selected endpoints using more replicates for suspected selective concentrations based on the initial exposure-response experiment.

2. Materials and methods

2.1. Set-up for biofilm growth in a flow-through system

To determine the selective properties of TC in complex aquatic bacterial communities, biofilms were established in aquaria containing different concentrations of TC. The experimental setup was designed with both high sensitivity and relevance in mind. Aquaria were constructed from molded glass (20 × 15 × 20 cm) with an outlet consisting of an inserted glass tube 5 cm from the bottom. Since glass is relatively inert, biofilms were grown on twenty microscope glass slides (VWR International, USA) held in place by a microscope slide staining rack in soda lime glass (Wheaton®, USA). Levels of nutrient as well as experimental time were optimized through several tests to ensure growth of a diverse biofilm at 20 °C in all TC exposures, which gave sufficient biomass for the endpoint analyses. Biofilms were studied because this is how most environmental bacterial communities grow. Furthermore, it provides a stationary phase (biofilm) in a continuous feed (planktonic phase) allowing for a longer selection time more representative of a long-term exposure as seen in affected environments. Notably, selection can take place on several levels, including effects on growth rate in the planktonic (seeding) and the biofilm communities, as well as selection of those bacteria that has an ability to attach, form and integrate into a biofilm in the presence of an antibiotic. In that sense, we here use the term MSC in a slightly wider meaning than the strictly growth-related definition used by Andersson and Hughes (2014). Since TC exposure was present during the establishment of the biofilm, all bacteria colonizing the slides were exposed, at least initially, without the protective properties of the extracellular polymeric substances (EPS) of a biofilm. Each aquarium had three different inlets consisting of inoculum (1 mL/min, through silicone tubing using PharMed 2.79 × 0.90 pump-tubing), nutrient (0.2 mL/min, in a sterilized flow system through silicone tubing using PharMed 2.54 × 0.90 pump-tubing) and TC (0.035 mL/min, through polytetrafluoroethylene (PTFE) tubing using PharMed 0.38 × 0.91 pump-tubing) supplied through peristaltic pumps (IPC-N pump; Ismatec AB, Switzerland). Inoculum was changed daily and consisted of treated sewage effluent (Ryaverket, Gothenburg,