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Selective concentration for ciprofloxacin resistance in *Escherichia coli* grown in complex aquatic bacterial biofilms

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ABSTRACT

There is concern that antibiotics in the environment can select for and enrich bacteria carrying acquired antibiotic resistance genes, thus increasing the potential of those genes to emerge in a clinical context. A critical question for understanding and managing such risks is what levels of antibiotics are needed to select for resistance in complex bacterial communities. Here, we address this question by examining the phenotypic and genotypic profiles of aquatic communities exposed to ciprofloxacin, also evaluating the within-species selection of resistant *E. coli* in complex communities. The taxonomic composition was significantly altered at ciprofloxacin exposure concentrations down to 1 μg/L. Shotgun metagenomic analysis indicated that mobile quinolone resistance determinants (*qnrD*, *qnrS* and *qnrB*) were enriched as a direct consequence of ciprofloxacin exposure from 1 μg/L or higher. Only at 5–10 μg/L resistant *E.coli* increased relative to their sensitive counterparts. These resistant *E. coli* predominantly harbored non-transferrable, chromosomal triple mutations (*gyrA* S83 L, D87N and *parC* S80I), which confer high-level resistance. In a controlled experimental setup such as this, we interpret effects on taxonomic composition and enrichment of mobile quinolone resistance genes as relevant indicators of risk. Hence, the lowest observed effect concentration for resistance selection in complex communities by ciprofloxacin was 1 μg/L and the corresponding no observed effect concentration 0.1 μg/L. These findings can be used to define and implement discharge or surface water limits to reduce risks for selection of antibiotic resistance in the environment.

1. Introduction

The rapid emergence and dissemination of antibiotic resistant bacterial pathogens is one of the most pressing threats to public health (WHO, 2014). Antibiotic resistance genes (ARGs) were, however, present in the environment long before antibiotics were introduced as clinical agents (D'Costa et al., 2011). Since then, many ARGs have been acquired and enriched in pathogens under a selection pressure from antibiotics. For example, the quinolone resistance gene *qnrA* is hypothesized to originate from the waterborne species *Shewanella algaeas* and has now spread to clinical isolates of different species within *Enterobacteriaceae* (Corkill et al., 2005; Poirel et al., 2005). Likewise, bacterial species occurring in the environment were identified as origins of the quinolone resistance gene *qnrB* and the carbapenem-hydrolyzing oxacillinase *OXA-181* (Jacoby et al., 2011; Potron et al., 2011). For most ARGs, however we know neither the origin nor the circumstances under which they were transferred to pathogens. Nevertheless, it is highly plausible that man-made antibiotics played and continue to play a critical role in this process.

Selection of antibiotic resistant bacteria can take place not only in or on the bodies of humans and domestic animals given antibiotics, but also in the external environment (Alonso et al., 2001; Martinez, 2008). Wastewater treatment plants (WWTP) have been identified as potentially important arenas for the emergence of new forms of resistance in pathogens (Rizzo et al., 2013). WWTPs are characterized by a coexistence of numerous intestinal commensals and pathogens, a wide

Abbreviations: ARG, antibiotic resistance gene; LOEC, lowest effect concentration; MIC, minimal inhibitory concentration; MSC, minimal selective concentration; NOEC, no effect concentration; QRDR, quinolone resistance-determining region; WWTP, wastewater treatment plant; OGLM, overdispersed poisson linear model

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diversity of environmental bacteria that may act as donors of novel resistance elements, and a mixture of antibiotics, antibacterial biocides and metals that all might contribute to selection of antibiotic resistant strains. We and others argue that the flow of ARGs from the vast environmental reservoir to pathogens must be managed to prolong the utility of antibiotics (e.g. Bengtsson-Palme and Larsson, 2015; Martinez et al., 2015). Hence, thorough knowledge regarding selective concentrations in the environment is urgently needed to guide mitigations in order to limit environmental selection of antibiotic resistance (Bengtsson-Palme and Larsson, 2018; Le Page et al., 2017).

Fluoroquinolones, including ciprofloxacin, are recognized by the WHO as critically important antibiotics for human medicine (WHO, 2017). The primary targets of fluoroquinolones are the bacterial type II topoisomerase DNA gyrase and topoisomerase IV, which are enzymes involved in the supercoiling of DNA and thus impair DNA replication (Drlica, 1999). Bacterial resistance against fluoroquinolones can arise from several mechanisms, including mutations in the genes encoding the targeted type II topoisomerases, increased drug efflux and transmissible target protection mechanisms (Redgrave et al., 2014). Fluoroquinolones are furthermore characterized by a high degree of persistence in the environment (Kümmerer et al., 2000; Golet et al., 2003; Cardoza et al., 2005). Concentrations found range from low ng/L to at the most a few μg/L as a result of excreted drugs from humans and animals (Golet et al., 2002; Zuccato et al., 2010; Bengtsson-Palme et al., 2016; Ory et al., 2016). In contrast, exceptionally high levels of ciprofloxacin (mg/L) and other fluoroquinolones have been found in effluent from drug manufacturers and in nearby, industrially polluted environments (Larsson et al., 2007; Fick et al., 2009; Kristiansson et al., 2011; Gothwal and Shashidhar, 2017). While it is indisputable that such high levels, way above the clinical breakpoint for most pathogens, will select for resistance, it is unclear what role much lower concentration might play, and hence where and to what extent mitigations are needed.

The relationship between the antibiotic concentration and its ability to select for resistance is, accordingly, critical to understand. Gullberg et al. used a test tube-based competition assay to determine the minimal selective concentration (MSC) of ciprofloxacin for *E. coli* (Gullberg et al., 2011). The lowest experimentally tested concentration that indicated a growth advantage of the *E. coli* harboring a resistance mutation in *gyrA* (S83 L) was 0.23 μg/L. Through extrapolation of growth data over a series of concentrations, they predicted that selection would potentially occur down to 0.1 μg/L. Consequently, a concentration below this predicted threshold would favor the sensitive variant and would not compensate for the growth impairment caused by the resistance mutation. This defined the MSC. Using a rather similar competition assay, studying the very same resistance mutation in *E.coli*, Liu et al. demonstrated selection for resistance at 3 μg/L, but not at 2 μg/L (Liu et al., 2011). Although it is not entirely clear why the studies differ quantitatively, it has likely to do with the more sensitive cell counting method used by Gullberg et al. (2011).

Notably, both of the above competition studies were performed under optimized laboratory conditions in a nutrient rich environment and with only two isogenic lab strains differing by one mutation competing against each other. Such studies likely give much valuable insights regarding the potential of antibiotics to select for resistant strains in environments characterized by low bacterial complexity and favorable growth conditions. Most microbial ecosystems, however, harbor many species competing for resources and the availability of nutrients varies considerably over space and time, placing quite different demands on a successful variant. Depending on the composition of the community, antibiotics may select for a range of chromosomally encoded resistance mutations as well as mobile genetic elements that may be transferred across strains and species, and a combination of both. Most importantly, selection in communities may occur primarily on the species level, with intrinsically resistant species filling the niches made available, rather than providing growth opportunities for strains with acquired resistance. Such between species selection could increase the abundance of species acting as donors of resistance factors. However, it is not as critical from a clinical risk perspective, as it would not per se contribute to the fixation of acquired resistance (Andersson and Hughes, 2011), nor would it generally increase the transmission possibilities of resistant pathogens (Pal et al., 2017). In a two-strain competition system, even costly resistance factors that confer a minor fitness advantage will take over the whole culture once a critical antibiotic concentration is reached (Andersson and Hughes, 2010). In a more complex community, other genotypes and species are more likely to fill up such a niche. In the environment, most bacteria exist in surface-associated multicellular communities, so-called biofilms. These are known to produce extracellular polysaccharides that on the one hand shape the biofilms architecture, and on the other hand cause a decreased permeability of toxicants, shielding sensitive species (Donlan and Costerton, 2002; Branda et al., 2005). The resistant biofilm population could also degrade an antibiotic and thus protect the more sensitive variants, as it was shown for TEM-1 β-lactamase expression in *E. coli* (Dugatkin et al., 2005; Perlin et al., 2009). Some bacteria are apparently capable of *catabolizing* synthetic fluoroquinolones (Dantas et al., 2008), but the nature of such enzymes is still unknown. So far, only quinolone *modifying* enzymes have been described, such as the aminoglycoside acetyltransferase AAC(6′)-Ib. Taking into account all of these aspects, selective concentrations derived from pairwise competition experiments may not be directly applicable to microbial communities.

Berglund et al. investigated the effect of a mixture of antibiotics, including ciprofloxacin (up to 20 μg/L nominal concentration) and norfloxacin (100 μg/L), on ARGs and integron prevalence using complex microcosms consisting of water and sediment from a Swedish lake (Berglund et al., 2014). Upon dosing the microcosm with antibiotics and following for 100 days, no increased prevalence of *qnrS* or the integrase gene *intI1* could be observed. However, already on the first day, measured concentrations of fluoroquinolones in the mesocosms were reduced between 90 and 95% from nominal concentrations, and they continued to decrease over time. Furthermore, the closed, nutrient poor experimental system used did likely not enable sufficient bacterial growth in order to detect small or moderate selective advantages of resistant bacteria.

To overcome these drawbacks, a continuous flow-through system was established to determine selective properties of antibiotic agents in complex aquatic bacterial biofilms (Lundström et al., 2016). This system enables studying the formation of complex bacterial communities in selection tanks where the only varying variable, the antibiotic concentration, can be controlled throughout the exposure period. By monitoring changes on phenotypic, genotypic and taxonomic levels, the selective potency of ciprofloxacin on a complex community was investigated. Despite the apparent high sensitivity, a limitation with the approach by Lundström et al. (2016) was that within-species selection was not studied specifically. Also, the setup is rather cost and labor-intense which restrict analysis with high throughput.

The aim of this study was to identify the Lowest Observed Effect Concentration (LOEC) of ciprofloxacin in a complex aquatic bacterial community, studying a range of endpoints that inform about risks for selection of resistance. These included taxonomic composition, within-species selection of resistant strains (*E. coli*), chromosomal resistance mutations, as well as transferrable resistance genes. This was studied in biofilms using sewage effluent inoculum and the flow-through system that has been described recently (Lundström et al., 2016), but we also made a brief comparison with a much less elaborate planktonic test tube culture with serial transfer of complex sewage effluent communities.